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AN AMPEROMETRIC ENZYME ELECTRODE FOR THE DETECTION OF L-LACTATE

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AN AMPEROMETRIC ENZYME ELECTRODE FOR THE DETECTION OF L-LACTATE

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ABSTRACT.

The main tasks of this thesis were to evaluate a number of amperometric enzyme electrode chemistries for the selective and sensitive detection of L-lactate, and apply mass fabrication technologies to reproducibly manufacture sensors in a controllable manner. The sensors studied were based on the use of lactate oxidase with a range of modified-carbon electrodes. Noble metals, hexacyanoferrate (III) or Prussian Blue were used to modify carbon electrodes for the electro-catalytic determination of hydrogen peroxide, the product of the reaction of lactate oxidase with L-lactate. Tetrathiafulvalene was employed as an artificial mediator between the enzyme and the electrode. Polypyrrole was tested as a means of immobilising lactate oxidase and to achieve direct charge transfer to the underlying carbon electrode.

The characteristics of the sensor responses to hydrogen peroxide, L-lactate and ascorbate were compared, in relation to the electrochemical electrode area. From this investigation, it was confirmed that screen-printed electrodes were more reproducible to manufacture than hand-fabricated electrodes. For screen-printed rhodinised-carbon electrodes, an operating potential of +400 mV (SCE) was selected. Interference from ascorbic acid and sensitivity to hydrogen peroxide were determined to be 26 $\mu A.mM^{-1}.cm^{-2}$ and 27 $\mu A.mM^{-1}.cm^{-2}$, respectively.

Screen-printed carbon electrodes modified with platinum, rhodium or palladium were selected for further investigation. Rhodium on carbon performed the best in terms of sensitivity and selectivity at low potentials, and different formations of rhodium-carbon complexes were studied. Although rhodium electroplated onto carbon screen-printed electrodes was examined, printing inks made from a preformed powder of rhodium on carbon-graphite proved to be the preferred route of electrode fabrication.

Screen printing, ink-jet printing and Cavro solution deposition were employed to fabricate the amperometric enzyme electrodes. These sensors were composed of rhodinised carbon and lactate oxidase in a water-based electrode ink with a protective outer membrane layer. Each stage, from ink preparation to membrane composition, was developed empirically. The sensitivity, stability and reproducibility of the working electrode was improved by altering it to a homogeneous ink, consisting of carbon graphite powder, rhodinised carbon powder (5% Rh by weight), hydroxyethyl cellulose (2% w/v) and lactate oxidase in the weight ratio of 2:8:18:1.

A layer of cellulose acetate (2% w/v in a 1:1 solution of acetone to cyclohexanone) and an outer coating of a polyurethane called Pellethane (1% to 4% w/v in dimethyl formamide and tetrahydrofuran) improved the selectivity, sensitivity and detection range of the sensor, allowing it to operate in physiological solutions with reduced passivation from protein adsorption.

The sensor design was revised to allow its passage through a catheter and operation within a blood vessel; it was manufactured on flexible material using screen printing and Cavro solution deposition techniques. These miniature sensors, with a working surface of 0.5 x 15 mm, were capable of linearly measuring lactate up to 3 mM in buffer solutions with an

average sensitivity of 44.8 nA.mM^{-1} L-lactate.

To test the sensor operation in physiological solutions, a flow injection system was employed. A planar three-electrode card used in this system was manufactured using screen printing and Cavro solution deposition techniques. L-lactate concentrations up to 6.4 mM were sensitively and, after minor correction, accurately determined in undiluted plasma and whole blood samples. This thesis has therefore made progress toward mass fabricating an amperometric enzyme electrode device suitable for the determination of L-lactate concentrations *in vitro*.

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CHAPTER 1:

GENERAL INTRODUCTION.

This chapter outlines the background to the use of amperometric enzyme electrodes for the measurement of blood lactate. Before investigating lactate detection, the significance and reason behind its measurement needs to be addressed. Additionally, in order to suggest ways in which methods of lactate determination can be improved, the current state of development needs to be reviewed. This general introduction surveys and assesses the limited methods available for the measurement of L-lactate in biological fluids in order to set objectives for the following research.

1.1. The Biochemistry of Lactate in Animals.

Lactate is an intermediary product of carbohydrate metabolism and produced mainly by muscle and red blood cells (Caraway & Watts, 1987; Kruse, 1993; Stryer, 1988). Glucose metabolism produces energy, carbon dioxide and water, and occurs in two stages. The first stage, producing a small amount of energy and pyruvate, takes place in the cytoplasm but the second stage takes place in the mitochondria. Here, in the presence of oxygen, a large amount of energy is produced when pyruvate participates in a cyclic reaction to form carbon dioxide and water. Under oxygen limiting or anaerobic conditions pyruvate accumulates in the cell and can inhibit the first stage. However, pyruvate can be converted to lactate by the enzyme lactate dehydrogenase, thus allowing the continuation of the first stage of glucose metabolism and producing some of the cell's requirement of energy. Lactate is a three carbon hydroxy acid that diffuses into the blood, and is present there as the lactate ion, as illustrated in Figure 1.1.

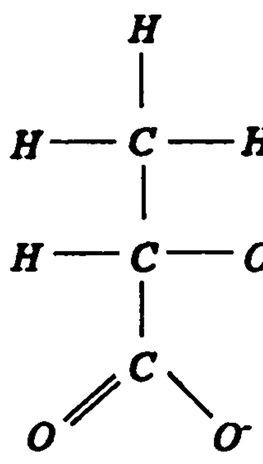


Figure 1.1. Structure of the L-lactate ion.

Many tissues can utilise lactate and the heart actually prefers it as a substrate, although 30% of the lactate produced by the body is metabolised in the liver. Lactate is first reformed into pyruvate which is then oxidised and liver and renal cortex tissues are capable of converting

lactate back to glucose (Caraway & Watts, 1976). Consequently, different lactate dehydrogenases are present in each tissue (Stryer, 1988). One isozyme (M_4, type 5) is found predominantly in liver and skeletal muscles and has a low affinity for pyruvate. This allows high concentrations of pyruvate to build up before being converted to lactate. Conversely, an isozyme which is inhibited by high concentrations of pyruvate and yet has a high affinity for it (H_4, type 1), is the principal form of lactate dehydrogenase found in the heart. It has been proposed that LDH in the heart is designed to oxidise lactate to pyruvate, which is then utilised as fuel, but is inhibited when there is limiting oxygen such as when high levels of pyruvate are present.

Thus, the formation of lactate allows continuation of energy production, whilst oxygen levels are low, and shifts some of the metabolic burden from muscle to liver tissue.

1.2. The Clinical Significance of Elevated L-lactate Levels.

A normal resting adult human will have an arterial blood lactate concentration of approximately 0.3 to 0.7 mM (Caraway & Watts, 1987; Siggaard-Andersen *et al.*, 1994). However, severe oxygen depravation of tissues (hypoxia) as in the case of muscle seizures, such as grand mal (epilepsy) or asthma, can result in the accumulation of lactate up to a concentration of 30 mM (Kruse, 1993). The underlying mechanism for this increase and accumulation of lactate is due to a decreased or inadequate systemic or regional oxygen availability and is termed type A lactic acidosis. There are two types of lactic acidosis, type A and type B (Kruse, 1993). Type B lactic acidosis, where lactate accumulation occurs without evidence of hypoxia, is associated with disease, drugs or toxins and congenital defects, and is divided into three subtypes:B_1, B_2 and B_3. It is not as common as type A. Examples of each type of lactic acidosis are listed in Table 1.1. The symptoms of acidosis are weakness, fatigue and finally coma.

Table 1.1. Clinical illness associated with lactic acidosis due to inadequate tissue oxygenation, disease, drugs, toxins or metabolic defects, classified by type A or B.

Causes of type A	**Causes of type B_1**	**Causes of type B_2**	**Causes of type B_3**
• Shock (circulatory or incipient) • Cardiac arrest • Profound hypoxemia • Carbon monoxide poisoning • Motor seizures	• Hepatic disease • Diabetes mellitus • Sepsis • Renal failure • Alcoholic ketoacidosis • Thiamine or iron deficiency • Short bowel syndrome • Meningitis	• Sodium bicarbonate • Fructose • Epinephrine • Norepinephrine • Cyanide • Salicylates • Acetaminophen • Ethanol, methanol • Ethylene or propylene glycol	• Deficiencies in enzymes of the phosphorylation pathway • Oxidative phosphorylation defects • Mitochondrial encephalomyopathy with lactic acidosis and stroke

Type A lactic acidosis has become expected as a complication of clinical illness. Lactate concentrations of 2.5 mM or higher have been considered clinically significant, indicative of possible tissue hypoxia or reduced blood flow and associated with increased mortality (Kruse, 1993). Levels of lactate in hospitalised patients in the range 4 to 10 mM signify extremely serious clinical conditions, above 10 mM indicates more than 90% probability of death (Cady *et al.*, 1973; Weil & Afifi, 1970). However, informing the clinician of an elevated blood lactate concentration can alert them to an early critical situation. This would allow them to instigate therapeutic measures before the levels of lactic acid became deleterious.

Clinical lactate measurement is an indication of oxygen debt, can guide the type and intensity of care, can help evaluate the response to therapy and is a prognosis of survival in critically ill patients with shock or multi-system disease.

1.3. The Significance of Elevated L-lactate Levels in Sports Medicine.

The rise in blood L-lactate concentrations during exercise was reported in the 1930's (Bang, 1936; Owles, 1930) and it is widely recognised that the regulation of blood lactate in athletes aids their performance (Billat, 1996; Friel, 1996; Janssen, 1987; Reilly *et al.*, 1990). Training not only increases the body's tolerance to working under anaerobic conditions but increases the removal of lactic acid. Although 2 mM L-lactate is more easily sustained, it has been suggested that training at an exercise intensity equivalent to a blood lactate concentration of 4 mM produces optimal adaptations. This training regime is based on the concept of 4 mM blood L-lactate representing the highest lactate steady state concentration that an individual can sustain and is known as the anaerobic threshold. Once the blood L-lactate concentration has reached 4 mM, it then accumulates quickly. A rise in blood L-lactate concentration to between 6 and 8 mM in athletes negatively influences co-ordination, seen as a drop in skill by sportsmen such as tennis players and ice-skaters, and can lead to injury. Lactate measurement has been a method for testing fitness for competition, designing training programs and evaluating athletes with poor performance.

The evaluation of blood lactate concentrations has also been related to racehorse fitness and training assessment (Davies & Pethick, 1983; Harkins *et al.*, 1993; Rainger *et al.*, 1994). Blood lactate accumulation above 4 mM under steady exercise is indicative of poor horse fitness or illness. Fast horses with a high power output incur rapid lactate accumulation and can only endure short distances, whereas slower horses with greater stamina show a gradual increase in blood L-lactate. To detect where the blood lactate concentration peaks so that performance and race distance may be determined, a series of blood samples rather than timed blood sampling should be performed (Evans *et al.*, 1993).

1.4. Measurements of L-lactate in Body Fluids.

There are three principal methods available for the analysis of lactate in biological solutions:

- chemical analysis;
- biochemical enzyme assay;
- dedicated analyser machinery.

Chemical methods include the conversion of lactic acid to insoluble zinc lactate which can

be estimated gravimetrically (Wolfe, 1914), oxidation of lactic acid in the sample with sulphuric acid to acetaldehyde which, when treated with *p*-hydroxydiphenyl and cupric sulphate, can be measured spectrophotometrically at 560 nm (Barker & Summerson, 1941) or oxidising the lactate with manganese oxide in acidic solution to acetaldehyde, distilling this into bisulphite solution and then iodometrically titrating the bound bisulphite (Olson, 1962). These are very old methods and have been superseded by easier and faster enzymatic determinations. There are several commercial spectrophotometric enzyme kits available, where an increase in absorbance is directly proportional to the L-lactic acid concentration of the sample. There are two principal methods.

Lactate dehydrogenase (E.C. 1.1.1.27, abbreviated to LDH), at high pH values in the presence of oxidised nicotinamide adenine dinucleotide (NAD^+), converts lactate to pyruvate:

$$L\text{-}lactic\ acid + NAD^+ \underset{\leftrightarrow}{LDH} Pyruvic\ acid + NADH$$

The reduction of NAD^+ to NADH can be monitored in the ultra-violet region of the spectrum at 340 nm. However, the equilibrium of the reaction is in favour of lactate and even under high pH conditions, lactate is not always fully oxidised. To displace the equilibrium in favour of pyruvate and NADH, pyruvate can be removed using hydrazine to form hydrazone (e.g. Sigma Diagnostics, Poole, Dorset, catalogue number 826-A (1997)). Alternatively, pyruvate can be trapped in an enzymic reaction involving L-glutamate and glutamate-pyruvate transaminase (GPT):

This method has been used by Boehringer Mannheim (Diagnostics and Biochemicals, Lewes,

$$Pyruvate + Glutamate \underset{\leftrightarrow}{GPT} L\text{-}alanine + 2\text{-}oxoglutarate$$

East Sussex) in their enzyme test kit for L-lactic acid (catalogue number 139 084 (1995)).

The other principle method involves the oxidation of L-lactate to pyruvate and hydrogen peroxide by using lactate oxidase (E.C.1.1.3.2., abbreviated to LOD):

$$L\text{-}lactic\ acid + O_2 \xrightarrow{LOD} Pyruvic\ acid + H_2O_2$$

Another enzyme, peroxidase, is present to catalyse the condensation of chromogen precursors to produce a coloured dye with an absorption maximum at 540 nm (e.g. Sigma Diagnostics, Poole, Dorset, catalogue number 735-10 (1997)).

Although it is possible to use these methods on a routine basis, analysers are becoming more frequently used, especially in clinical laboratories. They are very simple to operate and larger numbers can be analysed without labourious work. Bench-top L-lactate analysers such as those produced by Yellow Springs Instrument Company (Ohio, USA), NOVA Biomedical (Massachusetts, USA), Roche Bio-electronics (Basel, Switzerland), Analox Instruments Limited (London, UK) and, recently, Ciba Corning (Chiron Diagnostics, Halstead, Essex, UK) involve adding a small sample of blood and waiting for the result, and these are the most favoured amongst clinical technicians. However, for analysis outside the clinical laboratory, there are portable devices (produced by Analox, Yellow Springs and Boehringer Mannheim) which require only a small amount of blood with results given within minutes. The main analysers will be reviewed later to assess the performance of the device investigated and described in this thesis. Although there are several analysers presently available for the measurement of lactate in blood, all require blood samples to be taken and thus do not provide on-line measurements. There are currently no *in vivo* devices available for the measurement of L-lactic acid.

High performance liquid chromatography (HPLC) and Gas Chromatography/mass spectrometry have also been used to determine L-lactic acid concentrations in a variety of samples (Walker *et al.*, 1996; Willetts *et al.*, 1996; Yokoyama *et al.*, 1991) but these methods require expensive machinery and experienced operators.

1.4.1. Non-invasive Measurements.

Non-invasive measurements are helpful in cases where multiple serial samples are needed such as in sports medicine, and in the monitoring of children.

There is excellent correlation between blood and saliva lactate concentrations although the normal value in saliva (0.2 mM) is much lower than in blood (Altman & Dittmer, 1961). Whole saliva is a complex mixture of glandular secretions, leucocytes, sloughed epithelial cells and bacteria in crevicular fluid. The concentrations of most salivary constituents depend on the flow rate of saliva and hence, to obtain meaningful results, the collection of saliva needs to be standardised. Before analysing samples for lactate using spectrophotometric enzyme assays, the samples should be stored on ice to prevent alteration of the analytes and centrifuged to remove bacterial and other cellular debris. There have been reports of electrochemical sensors detecting the lactate levels in saliva, alleviating the need for centrifugation since turbidity is not a problem for this type of analysis (Guilbault *et al.*, 1995; Palleschi *et al.*, 1993; Palleschi *et al.*, 1991). These amperometric enzyme electrodes also measured the lactate rapidly, alleviating the need for sample storage and enabling fast serial determinations.

Sweat has been used by numerous workers as a non-invasive method of measuring L-lactate (Faridnia *et al.*, 1993; Guilbault *et al.*, 1995; Mitsubayashi *et al.*, 1994; Palleschi *et al.*, 1993; Taylor *et al.*, 1994; Vandam & Waterloh, 1983). High concentrations of L-lactic acid are present in sweat, approximately 30 mM at rest, and correlate well to the blood concentration (Altman & Dittmer, 1961). To obtain samples, subjects are thermally stimulated or required to exercise; the sample is collected by wiping the skin with absorbent material or a collection vessel. Although a little difficult to perform, this allows specific areas to be analysed. This approach may therefore be applicable to sports medicine but is not practical for the clinical ward in a hospital. It has been suggested that the lactate concentration is site specific and may include anaerobic production of L-lactic acid by the sweat glands in that region (Guilbault *et al.*, 1995; Taylor *et al.*, 1994).

Lactate concentrations in urine have been determined to identify metabolic or other disease in young children (Brook *et al.*, 1981; Castrogago *et al.*, 1995; Willetts *et al.*, 1996). It has also been suggested that urinary lactic acid measurements have a potential value for grading

birth asphyxia (Walker *et al.*, 1996). A bladder catheter can be inserted if a sample can not be obtained. The normal daily urine output of L-lactic acid is 5.5 – 22 mmoles (Caraway & Watts, 1987).

1.4.2. Invasive measurements.

In hospital operating rooms and intensive care units, invasive monitoring of patients is routine. Patients are often subjected to surgical incisions so invasive sampling does not pose a serious problem and it allows clinical information to be gained rapidly. Invasive sampling has also been applied in sports medicine where blood samples are taken from the ear-lobe or finger-tip (Kost & Hague, 1996).

The normal lactate concentration found in venous blood is 0.5 to 1.3 mM although hospitalised patients usually show a higher concentration range 0.9 to 1.7 mM L-lactate. Similarly, arterial blood normally contains 0.36 to 0.75 mM L-lactate but can rise to 1.25 mM under usual hospital conditions (Caraway & Watts, 1987). Collection of a blood specimen into tubes containing anti-clotting agents should be taken without a tourniquet from a resting patient, to prevent a rise in local lactate. Furthermore, specimens should be stored on ice and the blood lactate analysis should be carried out as soon as possible, to reduce the anaerobic metabolism of glucose that causes lactate concentrations to rise.

Instruments have been developed for whole blood lactate analysis (see Section 5.4.) but they often require an accurate manual or automatic dilution step. The presence of a variable packed cell mass can lead to serious errors in the calculation of dilution (Soutter *et al.*, 1978). Furthermore, dilution poses a particular problem in the case of lactate analysis due to the unequal distribution between the plasma and red blood cells and leaching can occur in diluted samples leading to a greater variation (Piquard *et al.*, 1980). Therefore, it is clear that to obtain a simple, rapid and accurate determination of blood lactate, it would be preferable to assay undiluted samples.

Plasma lactate values are approximately 7% higher than the concentration in blood and to obtain a specimen, a blood sample that has been chilled immediately after withdrawal, has to be centrifuged to separate the cells within 15 minutes (Caraway & Watts, 1987). The

procedure may vary between samples thus causing difference in lactate concentration, but once plasma is obtained, the lactate value remains constant for up to 8 days if appropriately stored at 4 to 8 °C. The commercial enzyme test kits available for lactate measurement require plasma samples since they operate optically by measuring the change in absorbance.

The concentration of lactic acid in cerebral spinal fluid usually parallels that of blood except where central nervous system disorders are present (Caraway & Watts, 1987). Cerebral spinal fluid, usually obtained by lumber puncture, has elevated levels of lactic acid with disorders such as meningitis, intercranial haemorrhage and epilepsy (Caraway & Watts, 1987; Dwivedi & Reddy, 1983; Kopetzky & Fishberg, 1933).

Whole-blood point-of-care testing and *in vivo* monitoring benefit cardiac and critical care where lactate monitoring of patients under anaesthesia or in intensive care is vitally important (Kost, 1993; Kost & Hague, 1996). By using an *in* or *ex vivo* monitoring device to acquire immediate information on the status of the patient, continuous surveillance can be achieved. Rapid and unexpected changes may be detected which would otherwise be missed by serial testing. The costs for a series of *in vitro* tests and the risk of transmission of blood-borne diseases are reduced.

1.4.3. Requirements of an *in Vivo* Device.

During the course of hospitalisation, most operating rooms and critically ill patients have indwelling arterial catheters (Kost, 1993; Kost & Hague, 1996). A catheter approach to *in vivo* measurement is thus favoured by clinicians. However, there are several challenges posed for this type of device which will be outlined below (Churchouse *et al.*, 1986; Claremont & Pickup, 1988; Kost, 1993; Kost & Hague, 1996; Rolfe, 1990; Silver, 1987; Wang, 1988).

An *in vivo* device is required to be both sensitive and selective towards the analyte of interest and produce a fast response (under two minutes). Linked to this is the ability to detect the analyte over the clinically useful range.

The size of the probe is an important consideration for an arterial or venous measurement.

It is suggested that the device should pass through a 20-gauge catheter perhaps along with other probes for the measurement of pH and oxygen tension and should therefore be less than 1 mm diameter, preferably less than 0.5 mm diameter. The shape and flexibility should also be modified to prevent excessive trauma and so that it doesn't compromise local blood supply or unintentionally press the device against the wall of the blood vessel. This would result in the lactate concentrations in the blood vessel wall being measured, not actually the blood lactate, and could accidentally puncture the blood vessel wall (Kost, 1993; Kost & Hague, 1996). The size and shape of an implanted object, as well as the chemical and physical nature of its surface, also contribute to an inflammatory response (Reach & Wilson, 1992).

Low toxicity of materials, immunogenicity, thrombogenicity and resistance to biodegradation are extremely important factors for an implantable device (Vadgama, 1992). The interaction of foreign materials with blood is an important process to be understood when developing implantable devices (Lelah & Cooper, 1986). Blood compatibility is complex and can be more easily defined in terms of what should not occur (Bruck, 1974), namely:-

- thrombosis;
- destruction of cellular elements (platelets, red blood cells, white blood cells);
- alteration of plasma proteins;
- enzyme destruction;
- electrolyte depletion;
- adverse immune response;
- damage to adjacent tissues;
- toxic or allergic reactions;
- activation of the complement system.

Physical phenomena, such as surface roughness, and chemical factors, such as wettability and surface charge, will affect the way in which the body reacts to the device. Membranes can impede these processes and provide a barrier to interferents, and must be selected carefully to provide these qualities.

Long term stability of the measuring capability of the device is necessary, as is the ability to calibrate the device *in situ*. An initial calibration along with occasional one or two point calibrations to check the measurement drift, is acceptable. A change in sensor output of less than 10% over 24 hours is acceptable for short term operation (Claremont & Pickup, 1988). For *in vivo* L-lactic acid measurement a device is needed to operate over 6 hours during surgery procedures or over a 24 hour period in intensive care units (Siggaard-Andersen *et al.*, 1994).

A pre-requisite for commercial production of implantable sensors should be their suitability to mass fabrication at low unit cost, at least on a medium batch scale, with appropriate quality assurance. By their nature, implantable devices have to be disposable but should also be easy to store with an adequate shelf life. They also need to be supplied ready for use within sterile packaging. The production of the device therefore has to keep sterility in consideration since polymer or metal materials are often affected by heat, chemical or radiation sterilization processes.

Finally, clinical acceptance is a major obstacle to be overcome when developing an implantable device. The instrumentation and sensing device need to be straightforward to use, safe and provide reliable information for diagnosis in therapy. Failure to gain clinical acceptance is usually due to inadequate overall performance.

1.5. Biosensors.

Biosensors are devices incorporating a biological sensing component combined with a transduction element that results in an electrical signal proportional to the analyte of interest (Karube & Yokoyama, 1993; Lowe, 1985; Scheller *et al.*, 1989; Turner *et al.*, 1989; Wang, 1988). Biosensors have met with some commercial success in the field of biochemical analysis because they perform sensitive and selective measurements on a real-time basis with the ability of cost-saving and automating routine analysis. Table 1.2. illustrates the diverse range of possible biosensors formed from combining biological recognition components with transduction elements.

Table 1.2. A range of detectable analytes alongside possible biosensor configurations.

Analytes	Biological Component and Sensor Type		Transducers
Metabolites	Enzyme	Biocatalytic	Electrochemical
Drugs	Whole cell	Biocatalytic	Optical
Micro-organisms	Organelle	Biocatalytic	Calorimetric
Viruses	Tissue slice	Biocatalytic	Piezoelectric
Antigens	Antibody	Affinity ligand	
Haptens	Cell receptor	Affinity ligand	
DNA	DNA	Affinity ligand	
RNA	RNA	Affinity ligand	

The specificity of enzymes coupled with their high turn-over rate provides a good component for catalytic biosensors. An important advantage of using electrochemical sensors over other transduction methods is their ability to be easily operated and the relative inexpensiveness of both materials and equipment. Additionally, minimal sample pretreatment is required and fast response times are achieved, leading to high throughput times. The optical properties do not affect the measurement whereas optical devices require a transparent or semi-transparent solution. An amperometric biosensor may be more attractive than a potentiometric device because of the generally higher sensitivity and wider linear range.

1.6. A Brief Description of Electrochemical Methods.

Electrochemistry describes the process involved when electrons are transferred between electrodes and reactants, which are usually in solid and liquid form, respectively (Bard & Faulkner, 1980; Fisher, 1996). The following parameters have been identified as controlling the charge transfer process at an electrode:-

- electrode potential;
- chemical nature of the electrode surface;
- structure of electrode-solution interface;
- transport of species between electrode and solution;
- reactivity of species involved.

1.6.1. The Electrochemical Cell.

A Galvanic cell converts the chemical activity into electrical energy whereas an electrolytic cell requires an external electrical driving force to cause a reaction to take place. The electrochemical reaction of interest takes place at the working electrode in an electrolytic cell. To measure this reaction, another electrode is needed: the reference electrode. The potential and current flow can then be measured across the working and reference electrodes when the potential drop in the solution is below 2 mV. Frequently, a third electrode is used. This ensures that a steady potential is held between the reference electrode and the working electrode when the solution is more resistive (say above 100 Ω), allowing large currents (above 10 μA) to be measured between the working electrode and the third electrode (auxiliary electrode). These experimental arrangements are schematically shown in Figure 1.2.

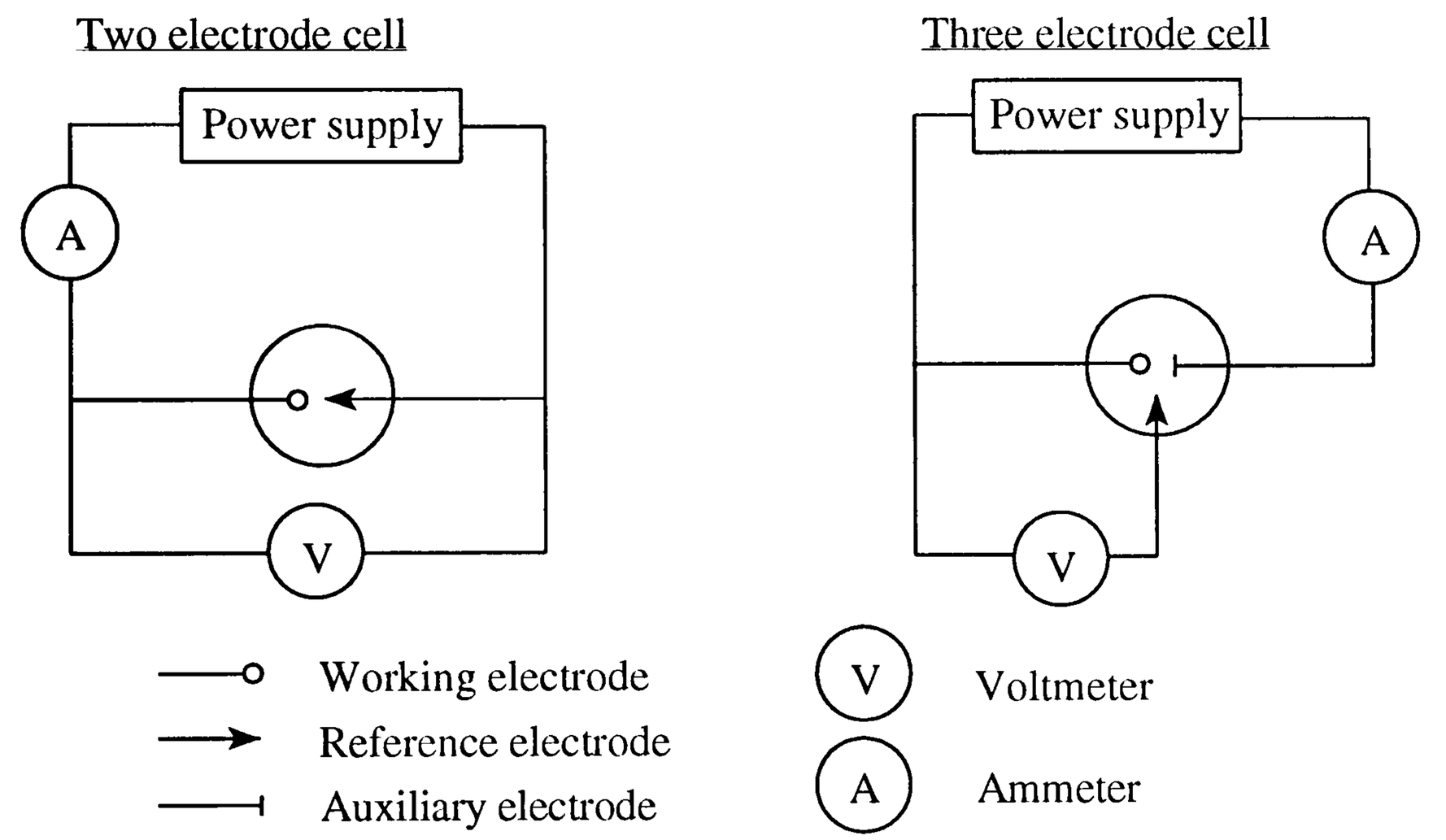


Figure 1.2. Arrangements for two and three electrode electrolytic cells.

1.6.2. Electrode Potential.

The potential applied between the reference and working electrode drives the electrochemical reaction: it causes the chemical species present in solution to be reduced or oxidised at the electrode's surface. It can be thought of as electron pressure; as the electrode potential becomes more negative, it becomes a stronger reductant (donates electrons) and as the potential becomes more positive, the electrode becomes a stronger oxidant (accepting electrons) (Wang, 1988).

1.6.3. Electrode Materials.

The reference electrode is made of non-polarisable material, that is, there is little change in the potential when a small current is conveyed. This ensures that the potential between the electrodes is stable because a charge does not build up on the electrode surface. A charge would affect the electrode-surface interface and lead to a potential difference. Conversely, to prevent any charge transfer across the electrode-solution interface, regardless of the applied potential, the working electrode is made of polarisable material. This ensures that only the reaction of interest is observed, the electrode does not react with the electrolyte.

1.6.4. Electrode-Solution Interface.

An equilibrium is established between the solution and an ideal polarised electrode such that the charges are equal but opposite. This electrostatic arrangement arises because no transfer of charge can take place between the electrode and the solution and yet the interface as a whole needs to maintain neutrality. The electrode-solution interface is analogous to a capacitor, charge will build up at the interface until an equilibrium is achieved which is dependent on the potential applied. The whole array of charged species and orientated dipoles existing at the metal-solution interface is called the electrical double layer (Bard & Faulkner, 1980; Fisher, 1996).

The currently accepted model of the electrical double layer is shown in Figure 1.3., where the interface is comprised of three layers. The first layer, the inner Helmholtz plane, is composed of specifically adsorbed species and solvent molecules. The second layer, the outer Helmholtz plane, consists of solvated ions attracted to the electrode surface but are unable to move any closer due to their hydration shell. Finally, the diffuse layer of solvated molecules is considered to be in equilibrium between the outer Helmholtz plane and the random forces of the bulk solution.

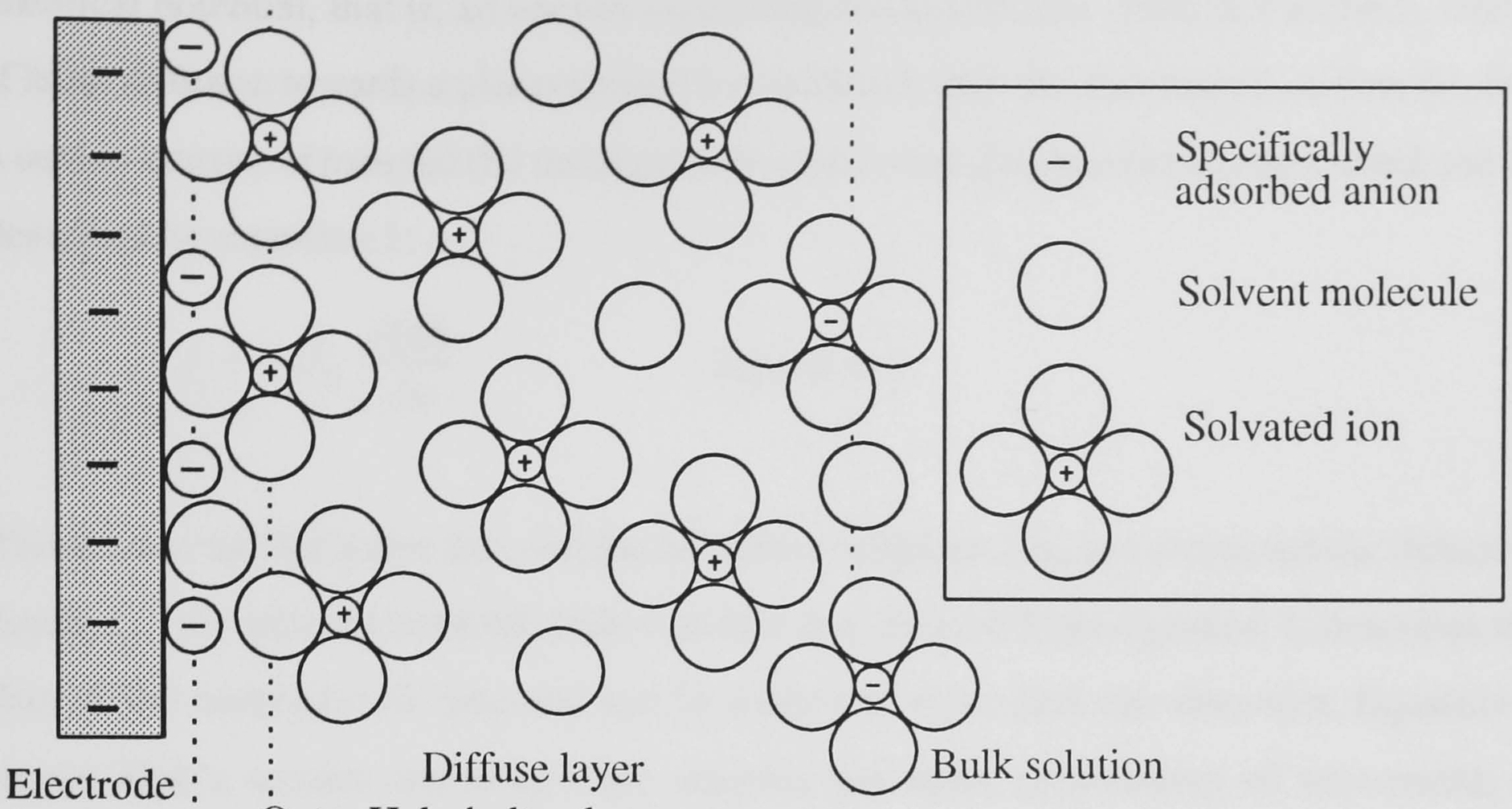


Figure 1.3. Grahame's model of the electrode-solution interface showing the three regions of attraction. Electrode shown with a negative charge.

1.6.5. Mass Transport.

The transport of species between the electrode and solution is described by three significant processes, namely: convection, migration and diffusion.

Convection is stirring or hydrodynamic transport (Bard & Faulkner, 1980). It consists of both natural and forced convection. Natural convection is caused by thermal and/or density gradients and becomes significant at macro electrodes after approximately 10 s. Forced convection can take the form of stirring, pumping or bubbling gas through the solution and can be applied to swamp contributions from natural convection to make experiments over 10 s long more reproducible.

The movement of a charged body under the influence of an electrical field is known as migration (Bard & Faulkner, 1980). The mobility of the charged body (ion) is dependent on its charge and size, as well as the viscosity of the solution. Migration effects on the analyte of interest are usually lowered by adding electrolytes: small ions of opposing charge which have the same relative mobility.

Diffusion is described as the movement of species under the influence of a gradient of chemical potential, that is, an uneven concentration distribution (Bard & Faulkner, 1980). If linear diffusion towards a planar surface is considered, then the movement, or flux (j), that a certain amount of material (B) undergoes through a unit distance (x) in one second can be described by equation 1:

$$j = -D_O \frac{\partial[B]}{\partial x} \qquad \text{Equation 1.}$$

This is know as Fick's first law, and the diffusion coefficient, D_O, is a characteristic diffusion function of the material involved. Fick's second law, derived from equation 1, describes the flux of the material over time and can be related to more than one direction. Equation 2 shows Fick's second law where the material has three components of movement, in directions x, y and z:

$$\frac{\partial[B]}{\partial t} = D_O \frac{\partial^2[B]}{\partial x^2} + D_O \frac{\partial^2[B]}{\partial y^2} + D_O \frac{\partial^2[B]}{\partial z^2} \qquad \text{Equation 2.}$$

However, to use these equations, the experimental conditions need to be defined. The Cottrell equation is easily used and defines the current density I, at a planar electrode in an unstirred solution containing excess electrolyte at constant temperature. This is shown in equation 3, where n is the number of electrons, F is the Faraday constant, D_O is the diffusion coefficient of species B, A is the electrode area, $[B]$ is the concentration of B in the bulk solution and t is the time:

$$I = -nFD_O^{½}A \frac{[B]}{t^{½}\pi^{½}} \qquad \text{Equation 3.}$$

1.6.6. Reactivity of Species.

The balance of the concentrations of reactant and product at any potential is kept in equilibrium with their formal potential described by the Nernst equation:

$$E = E^\circ - \frac{RT}{nF} \ln\frac{[O]}{[R]}$$

The formal potential is the potential at which the activity of the oxidised and reduced form

of the species is equal. The activity is related to the concentration by the activity coefficient.

1.6.7. Voltammetric Techniques.

Electrochemical methods are employed as tools in the study of chemical systems, the following voltammetric techniques were used in this thesis.

1.6.7.1. Cyclic Voltammetry.

Cyclic voltammetry is the measurement of current with respect to a changing potential where the potential is swept from potential E_1 to E_2 and back to E_1 at least once. Measurements usually employ the three electrode cell and take place in a quiescent solution. Cyclic voltammetry is mainly used as a qualitative method for the characterisation of redox species in solution. The scan rate, peak potential and peak current can provide quantitative information regarding diffusion, adsorption and the type of reaction occurring, leading to the determination of the rate constant under certain conditions (kinetic data).

If a forward scan towards more positive potentials is considered with a reaction:

$$R \dashrightarrow O + ne^-$$

then current responses are observed as shown in Figure 1.4. Initially the current increases until a plateau is achieved, at slow scan rates. This is the steady state response. At faster scan rates, the current-potential curve becomes more peaked shaped. By considering the

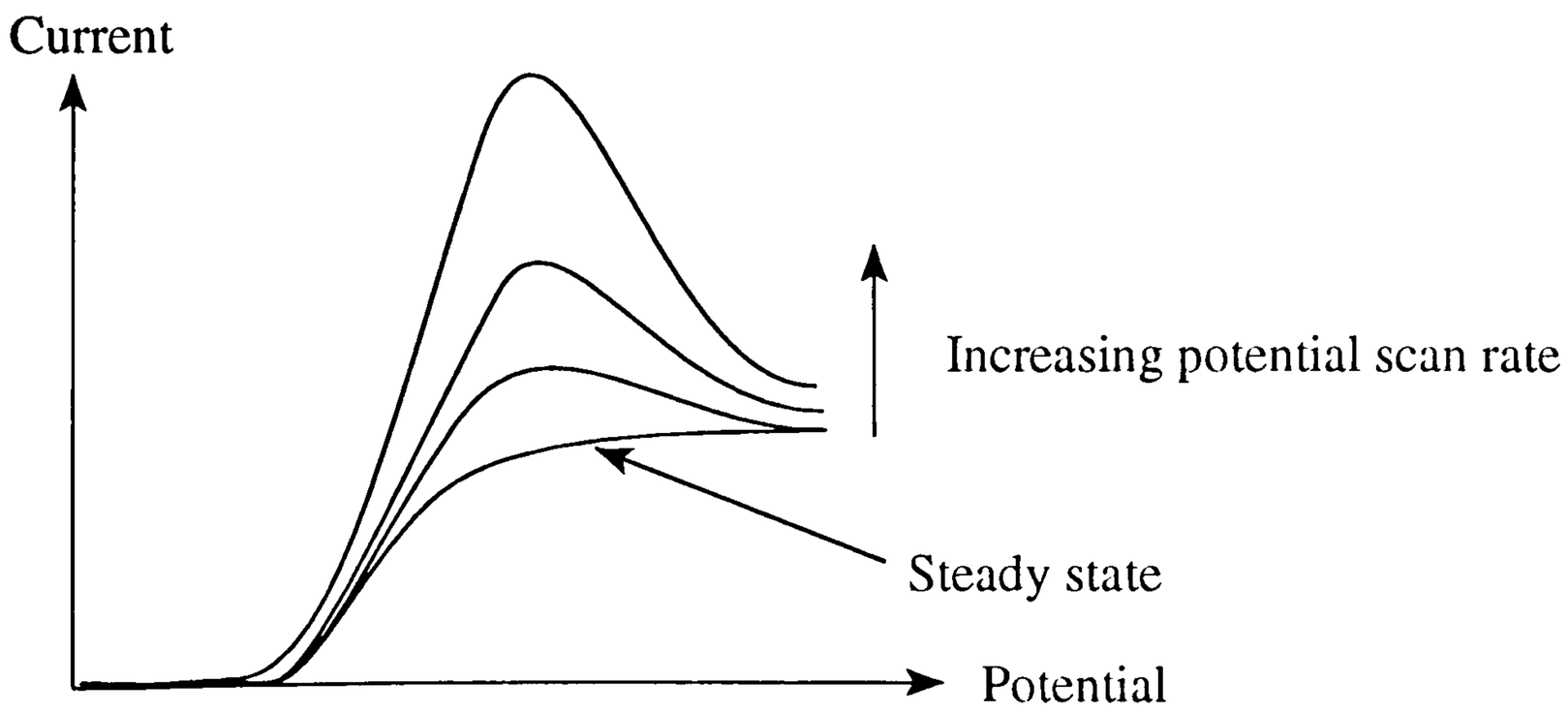


Figure 1.4. A series of linear sweep voltammograms for the oxidation of species at several potential scan rates.

Nernst equation and the changes in concentration that occur in the solution adjacent to the electrode during electrolysis, this relationship can be explained (Kissinger & Heineman, 1983). In a solution containing only species R, the concentration of R and O at any potential is kept in equilibrium, described by the Nernst equation. As the potential is altered, the equilibrium adjusts. At potentials higher than the formal oxidation potential, the concentration of O builds up at the electrode surface. A concentration gradient then develops, driving O away and R towards the electrode surface. This flux of material is associated with an increase in current. At a slow scan rate the rate of diffusion is proportional to the conversion of R to O. However, at higher scan rates, a steeper concentration gradient is formed which increases the rate of flux. This in turn increases the current flow. As the potential increases further, less R is available since the rate of diffusion to the surface is not as great as the reaction, therefore the concentration gradient relaxes and causes the flux to decrease. The reduced flux corresponds to the declining slope of the peaked response.

The reverse scan would also elicit a peaked response if the reaction was reversible and followed the Nernst reaction. There are two further considerations:

1) the reactant and the product should be stable in solution;

2) the rate of electron transfer should be faster than the rate of diffusion.

This would give rise to the cyclic voltammogram similar to Figure 1.5:-

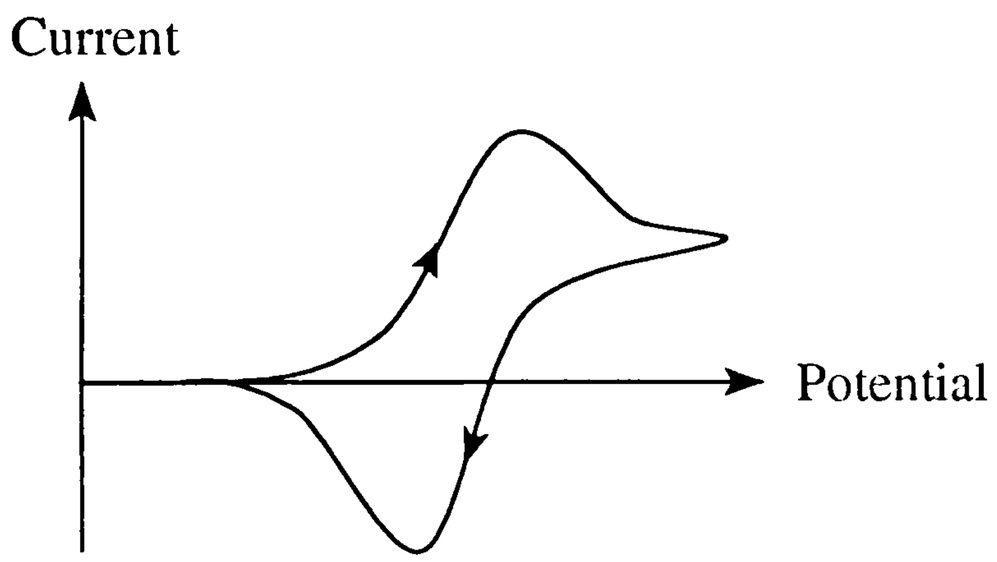


Figure 1.5. Typical cyclic voltammogram of a reversible reaction.

If the reactions are quasi-reversible or irreversible due to the rate of mass transport being greater than the rate of electron transfer or the species not being chemically or physically stable, then the cyclic voltammogram would change. This would take the form of the peaks

separating more than 59 mV, one peak may be missing or the peak heights would not increase upon increased scan rate.

1.6.7.2. Chronocoulometry.

Coulometry involves the measurement of the charge passed across an electrode. A charge of one Coulomb is the amount of current passed when one mole of electrons is transferred in one second. Therefore chronocoulometry measures the total charge passed over a certain time period, allowing the amount of reactant to be determined. A potential is stepped from an initial value where no reaction occurs, to a value where the reaction occurs at a maximal rate (a diffusion controlled rate) and the passing charge measured. In an unstirred solution, the charge with respect to time on the application of an oxidising potential can be described by the integrated Cottrell equation:

$$Q = \frac{2.n.F.A.D_O^{½}.[B].t^{½}}{\pi^{½}} + Q_{dl} + n.F.A.\Gamma$$

where A is the area of the electrode surface, Q_{dl} is the charge due to the double layer capacitance and the term $n.F.A.\Gamma$ is the charge contribution of the reduction of adsorbed species on the electrode surface (Bard & Faulkner, 1980). The charge contributions from the double layer and the adsorbed species are not time dependent and rapidly decrease, they are represented by the intercept on the charge axis of a charge versus square root of time plot. This plot is commonly used to determine the diffusion coefficient of species B. Alternatively, the surface area of the electrode can be calculated if the diffusion coefficient of the species is known, as demonstrated in Section 2.3.6. To prevent convection significantly affecting the accumulating charge of the unstirred solution, measurements are usually taken within the first ten seconds. Additionally, rapid depletion of species in the diffusion layer adjacent to the electrode surface may occur, reducing the measurement time further.

1.6.7.3. Amperometry.

Amperometry is the measure of current at a certain potential with respect to time. A long time scale is usually used and therefore, to swamp natural convection, the solution is stirred. The steady state current at a known potential is measured, giving rise to a quantitative

measurement of current which is proportional to the concentration of analyte in solution. The following relationship between the current passed and the concentration of analyte has been solved from Fick's second law, where δ is the diffusion layer thickness:

$$I = -nFAD_O\frac{[B]}{\delta}$$

1.7. Amperometric Enzyme Electrodes for L-lactate.

By combining the specificity of a biological enzyme with the sensitivity of an amperometric electrode, Clark and Lyons constructed the first re-usable biosensor in 1962 (Clark & Lyons, 1962). This amperometric enzyme electrode combined the highly specific glucose oxidase enzyme, entrapped by a semi-permeable membrane, with an oxygen electrode. It detected glucose concentrations via the enzymic uptake of oxygen, producing gluconic acid and hydrogen peroxide from glucose. The research into glucose biosensors has motivated the investigation into sensitive and reusable L-lactate sensors. There are currently four enzymes used in the construction of amperometric biosensors for the determination of L-lactate.

1.7.1. Lactate Dehydrogenase.

This enzyme, also known as NAD-dependent lactate dehydrogenase (E.C.1.1.1.27), converts lactate to pyruvate as described earlier in Section 1.4. It is commercially available from many sources as either a powder or solution with various activities towards lactate, depending on the source as well as the mixture of isozymes present (as outlined in Section 1.1.).

A number of enzyme electrodes and detectors have been constructed for the determination of L-lactate based on the electrochemical regeneration of NAD^+(e.g. (Wang & Chen, 1994b; Yamanaka & Mascini, 1992; Yao *et al.*, 1982). The cofactor NAD^+ is not chemically bound to lactate dehydrogenase and can therefore leech from the electrode, rendering a lower and irreproducible signal. Moreover, to fully exploit the advantages of a biosensor over alternative chemical analysis, it has to be reagentless. Problems are also encountered when oxidising nicotinamide adenine dinucleotide; electrodes are passivated due to by-products of the oxidation reaction that foul the surface, and oxidisable species present in the sample produce interfering signals due to the high potential applied. There have been many attempts

to overcome these problems, as mentioned briefly below.

By using mediating compounds that readily oxidise NADH to NAD^+ and can be easily regenerated at low electrode potentials, interference from oxidisable species can be lowered. Malinauskas and Kulys (1978) studied the use of phenazine ethosulphate (PES^+) as an electron carrier between NADH and oxygen in aqueous solution to detect lactate. Vadgama *et al.* (1986) investigated both the oxygen uptake and the hydrogen peroxide generation using this mediation method with lactate dehydrogenase but found that the stability of PES^+ under highly alkaline conditions (necessary to drive lactate oxidation) was poor, precluding its practical use. They were, however, able to measure blood lactate concentrations using the electrochemical detection of hydrogen peroxide.

Methylene blue has also been used to mediate between NADH and the electrode. It has been used with lactate dehydrogenase immobilised in polypyrrole on a glassy carbon electrode to detect lactate at 0 V (SCE) (Karyakin *et al.*, 1994b) and on a platinum electrode to detect lactate linearly up to 3 mM at +300 mV (Ag/AgCl) (Ikariyama *et al.*, 1990).

Meldola's blue has been added to a carbon paste electrode containing nano-particles of fumed-silica (Wang & Liu, 1993). The fumed silica alone catalytically regenerated NAD^+ which was added with lactate dehydrogenase to the carbon paste electrode. It was reported that the adsorptive properties of the large surface area of the nano-particles of fumed-silica aided retention of the constituents; 85% of the Meldola's blue response was retained after numerous repetitive scans which would normally have deteriorated after 12 scans. Recently, Meldola's blue, NAD^+ and lactate dehydrogenase have been combined in a carbon based screen-printing ink to produce disposable biosensors which could measure lactate in small volumes of serum at low working potentials around 0 V (Ag/AgCl) (Sprules *et al.*, 1996; Sprules *et al.*, 1995). Although NAD^+ was not chemically bound to the enzyme electrode, it remained in close proximity of the electrodes, which were intended for single use.

Printing has also been applied to fabricate lactate dehydrogenase based lactate sensors with catalytic surfaces. Silber *et al.* (1994) used prefabricated gold thick-film electrodes onto which enzyme loaded membranes were dispersed using a semi-automated dispenser system.

The electrodes were used at +600 mV (Ag/AgCl) in a flow stream, to which NAD^+ was added, to determine the lactate concentration of milk samples diluted 1:50 in buffer. Two samples were sequentially tested, the second including NAD^+ so that the response from the first sample could be subtracted to eliminate interfering signals. Yoon and Kim (1996) immobilised lactate dehydrogenase and NAD^+ in an activated carbon printable ink and detected lactate at +350 mV (Ag/AgCl). The performance of activated carbon was improved over glassy carbon and carbon paste electrodes for the oxidation of NADH, which occurred at +595 and +620 mV (Ag/AgCl), respectively. They also discovered that silicone oil, added to the ink, improved the reproducibility of the response by retaining the NAD^+ for a longer period of time.

By electrochemically pretreating glassy carbon electrodes using cyclic voltammetry, Cenas *et al.* (1984) were able to detect lactate at +0.2 V (Ag/AgCl) by immobilising lactate dehydrogenase under a cellulose dialysis membrane with NAD^+ in the buffer solution at both pH 8.8 and 7.5. They showed that the sensitivity did not change considerably with time at pH 8.8 but did at pH 7.5, due to enzyme deactivation. The electrochemical pretreatment may have formed quinoidal groups which react with NADH at a lower working potential. Carbon black provides quinoidal groups to catalytically oxidise NADH at potentials around 0 V (SCE). This was exploited by Kanapene *et al.* (1992) to detect lactate at a glassy carbon electrode with lactate dehydrogenase immobilised on a polyamide membrane.

Lactate dehydrogenase has been used in conjunction with other enzymes in an attempt to lower the working potential and enhance the current response. Diaphorase can be used to convert NADH to NAD^+ using potassium hexacyanoferrate (III) in the following manner:

$$NADH + 2Fe(CN)_6^{3-} \xrightarrow{diaphorase} NAD^+ + 2Fe(CN)_6^{4-} + H^+$$

where the hexacyanoferrate (III) is regenerated at a platinum electrode. Yao and Wasa (1985) packed a column with silica gel onto which lactate dehydrogenase was immobilised by glutaraldehyde. This was linked with a platinum electrode modified with diaphorase in a flow stream and a working potential of +0.4 (Ag/AgCl) was applied to detect D- and L-lactate in buffer solution at pH 9.5, depending whether the D- or L- lactate dehydrogenase

was used. The column was still 90% active after two months. Durliat *et al.* (1990) also used the diaphorase system with potassium hexacyanoferrate. A semipermeable cellophane membrane kept the enzymes and hexacyanoferrate in close proximity to the platinum electrode, poised at +0.3 V (SCE), allowing the NAD^+ and analytes to diffuse through from the buffer.

The enzyme glutamate-pyruvate transaminase (GPT) has been used in both column and electrode devices to trap pyruvate and force the reaction to completion when glutamate is added, as described in Section 1.4. (Gorton & Hedlund, 1988; Hikima *et al.*, 1995). Gorton and Hedlund (1988) inserted a spectroscopic carbon rod modified with Meldola blue into a flow cell down stream from an enzyme column and electro-catalytically detected NADH at 0 V (Ag/AgCl). However, they suggested using other more stable mediators. Hikima *et al.* (1995) immobilised both LDH and GPT on a Biodyne membrane placed on a glassy carbon electrode and used pulsed amperometry to satisfactorily detect L-lactate in serum samples, within experimental errors. Although NAD^+ was still added to the glycine buffer (pH 9.5) and not immobilised at the electrode, this technique had the advantage of a higher sensitivity and selectivity in comparison to the classical direct-current amperometric detection and lactate could be detected at the low potential of -1.08 V (Ag/AgCl).

Many attempts have been made to construct amperometric L-lactate sensors using LDH, but the problems associated with the use of this enzyme have not been sufficiently resolved to provide adequate probes for continuous L-lactate measurement.

1.7.2. Flavocytochrome b_2.

L-lactate: cytochrome c reductase (E.C.1.1.2.3.), known as flavocytochrome b_2, oxidises lactate to pyruvate and transfers the charge to an electron acceptor such as cytochrome c. It was first extracted from *Saccharomyces cerevisiae* (baker's yeast) but a more active and stable form can be extracted from the yeast *Hansenula anomala* (Chapman *et al.*, 1991). Flavocytochrome b_2 is commercially available in solution (e.g. lactic dehydrogenase product number L 4506, Sigma Chemical Company, Poole, Dorset) with a general activity of 0.1 to 0.6 Units per milligram of protein. One Unit of the enzyme will reduce 1 μmole of hexacyanoferrate (II) and will oxidise 0.5 μmoles of L-lactate to pyruvate per minute at pH 8.4 and 37 °C.

An early blood lactate analyser designed for discrete measurements at the bedside, the Lactate Analyser 640 (Roche Bio-medical, Basel, Switzerland), employed flavocytochrome b_2 immobilised in a cellophane membrane selectively permeable to lactate, pyruvate and the mediator hexacyanoferrate (III). The membrane was held on to a platinum electrode poised at +350 mV (Ag/AgCl) to regenerate hexacyanoferrate (III) (Racine *et al.*, 1975). Extracellular lactate could be measured from 0 to 12 mM but false results were given for blood measurement because the sample had to be diluted and the packed cell volume was not taken into consideration (Soutter *et al.*, 1978). The collection method was later improved in 1979 to lyse blood cells, although Piquard *et al.* (Piquard *et al.*, 1980) suggested that the blood lactate concentration was then increased and recommended that the blood sample should be directly analysed within 10 minutes to prevent excessive increases.

Although hexacyanoferrate (III) is the most common electron acceptor used with flavocytochrome b_2 (Chapman *et al.*, 1991), other mediators have been used to construct amperometric biosensors for L-lactate. These include tetrathiafulvalene (TTF) (Bartlett & Caruana, 1994) and complexes of N-methylphenazinium (NMP+) with the anionic radical 7,7,8,8-tetracyanoquinodimethane (TCNQ) (Kulys, 1986; Kulys & Svirmickas, 1980). Whole *Hansenula anomala* yeast cells have been combined with several different mediators in carbon paste, and it was suggested that flavocytochrome b_2 was providing the biocatalytic action (Kulys *et al.*, 1992b). The mediators included methylene green, Meldola's blue, N,N,N',N'-tetramethyl-1,4-phenylenediamine with TCNQ (TMPD/TCNQ), phenazine methosulphate, hexacyanoferrate and some combinations. Their activities were compared and it was found that hexacyanoferrate was the most sensitive, although phenazine methosulphate was active enough to be used with the yeast and carbon paste matrix to detect lactate concentrations in serum at +0.1 V (SCE).

A carbon paste electrode combining flavocytochrome b_2 with the natural electron acceptor cytochrome c and a mixture of lipids that mimic the biological membrane environment (asolectin), was also used to detect lactate, at +0.15 V (SCE) (Amine *et al.*, 1994). Cytochrome c alone does not usually exhibit reversible electrochemical behaviour at conventional electrodes, but by mixing with lipid-containing carbon paste, its electro-activity

could be followed using cyclic voltammetry.

The respiratory chain from *Escherichia coli* was immobilised in gelatin and fixed onto an oxygen electrode. This probe measured the uptake of oxygen to selectively determine the concentration of L-lactate in diluted wine and yoghurt samples and blood (Adamowicz & Burstein, 1987). It was suggested that lactate was oxidised by flavocytochrome b_2 present in the respiratory chain.

Flavocytochrome b_2 has also been used to directly interact with carbon based electrodes to detect lactate in human plasma and cell cultures (Staskeviciene *et al.*, 1991). It was thought that surface quinoidal groups present on the carbon black modifier were responsible for re-oxidising flavocytochrome b_2 after its conversion of lactate to pyruvate.

1.7.3. Lactate Monooxygenase.

Lactate monooxygenase (LMO) from *Mycobacterium smegmatis* (E.C.1.13.12.4), also known as lactate oxidase, is a bacterial flavoenzyme which catalyses the oxidation of L-lactate in the presence of oxygen to acetic and carbon dioxide. Pyruvate can be formed as an intermediate which dissociates from the enzyme in the absence of oxygen (Lockridge *et al.*, 1972). It is known that high phosphate concentrations can competitively inhibit the enzyme which therefore prevents its application for measuring L-lactate in physiological samples (Wang & Heller, 1993).

Lactate monooxygenase coupled with an oxygen electrode in various configurations have been used to detect L-lactate by the amount of oxygen uptake. Mascini *et al.* (1984) immobilised LMO on a nylon net over an oxygen electrode to form a simple lactate sensor that detected lactate in plasma, diluted in citrate buffer. The citrate buffer prevented inhibition by phosphate and other anions like oxalate, hydrogen carbonate and chloride present in blood. Makovos and Liu (1985) used LMO in saline solution which prevented reuse of the enzyme. They found the optimum pH range to be 5.6 to 6 and an excellent linear relationship on a log-log scale between 0.3 and 25 mM lactate in the temperature range 8 to 34.5 °C. Weaver and Vadgama (1986) used lactate monooxygenase entrapped in an ultrafiltration membrane retained over an oxygen sensor housed in a flow chamber to

continually analyse samples of serum, plasma and blood. They noted that heparin-fluoride was used as a blood anticoagulant because of oxalate inhibition of the enzyme. Lactate monooxygenase entrapped in a gelatin membrane over the surface of an oxygen sensor was used to determine lactate by Weigelt *et al* (1987) and was later developed to detect a range of substrates by co-immobilising other enzymes (Scheller *et al.*, 1988). Lactate monooxygenase and lactate oxidase were co-immobilised by Campanella *et al.* (1993) but the hydrogen peroxide from lactate oxidase was measured, the LMO was only used to extend the linear detection range. Although the system was used and stored in phosphate buffer, there was no report of inhibition.

Whole cells from *Acetobacter pasteurianus* retained on nylon membrane have been used in combination with an oxygen electrode to detect L-lactate in yoghurt and milk samples (Luong *et al.*, 1989). It was suggested that the uptake of oxygen occurred whilst lactate was converted to acetate via lactate mono-oxygenase enzyme found in the bacteria.

1.7.4. Lactate Oxidase.

The majority of research carried out on amperometric lactate sensors has involved the use of lactate oxidase. The accumulated knowledge of the construction of glucose sensors, based on glucose oxidase, has heavily influenced the development of amperometric lactate oxidase enzyme-electrodes (Clark *et al.*, 1984; Mullen *et al.*, 1986). Not only is lactate oxidase highly active towards L-lactic acid but it is commercially available as a relatively stable lyophilised powder amenable to different applications. Lactate oxidase converts L-lactate and oxygen into pyruvate and hydrogen peroxide, as described in Section 1.4. It does not require the addition of chemical co-factors, is not inhibited by compounds found in physiological solutions and is stable at physiological pH.

1.7.4.1. Lactate Measurement Based on the Detection of Oxygen Uptake.

A number of sensors have been described for L-lactate determination in serum (Hakanson *et al.*, 1993; Mizutani *et al.*, 1983) and blood (Baker & Gough, 1995; Kyrolainen *et al.*, 1993; Mascini *et al.*, 1985) based on the measurement of oxygen consumption by lactate oxidase immobilised over an oxygen electrode. Although the decrease in oxygen was monitored, there is concern that the additional consumption of oxygen by the amperometric

electrode in an anaerobic blood sample would lower the enzyme response and hence reduce the signal. The implantable device described by Baker and Gough (1995) accommodated for this by measuring the local oxygen concentration and correlating the lactate signal to the oxygen concentration using a mathematical manipulation. Their results indicated that the sensor could detect to at least 8 mM L-lactate in a solution with oxygen content of 0.02 mM (15 mmHg oxygen partial pressure) and at the normal concentration of oxygen (approximately 0.06 mM oxygen or 40-45 mmHg oxygen partial pressure) the sensor could detect up to 25 mM L-lactate.

1.7.4.2. Lactate Measurement Based on Hydrogen Peroxide Detection.

Instead of measuring the consumption of oxygen, the generation of hydrogen peroxide has frequently been measured as a means to monitor oxidase enzyme reactions. A number of reports have described the use of lactate oxidase in membranes placed over a platinum working electrode poised between +600 and +700 mV (Ag/AgCl) in a flow system. An early report by Clark *et al.* (1984) described the adaption of the Yellow Springs Instrument model 23A (YSI, Yellow Springs Instrument Company, Ohio, USA), developed for glucose analysis, for the determination of lactate in whole blood. They incorporated lactate oxidase between an inner cellulose acetate membrane and an outer polycarbonate membrane which was placed over a platinum electrode mounted in the YSI analyser. Small volumes of blood (10 to 20 μl) entered the reaction chamber and were diluted 1 in 24 with buffer (for the 10 μl sample), to measure the lactate content.

Since this early work, lactate oxidase has been immobilised in or on various membranes placed over a platinum working electrode to determine lactate in dairy products (Bardeletti *et al.*, 1986; Haketa *et al.*, 1990) and cell cultures (Renneberg *et al.*, 1991; Tsuchida *et al.*, 1985) but the majority of work has focussed on blood lactate determination. The lactate concentration in undiluted blood in a flow stream was monitored by Mullen *et al.* (1986) by using a combined membrane structure over a platinum electrode to reduce interferents and extend the detection range. Petersson (1988) used the YSI lactate oxidase membrane in a computer-controlled flow-injection system to determine the lactate concentration of whole serum and Meyerhoff *et al.* (1993) used the YSI lactate oxidase membrane over a platinum electrode for *ex vivo* analysis of whole blood. The sensor was held down-stream from a

double lumen catheter that diluted the blood 1:3 in heparinised physiological saline. Other membranes used to immobilise lactate oxidase over a platinum electrode in a flow system for blood analysis include polyurethane (Pfeiffer *et al.*, 1993; Pfeiffer *et al.*, 1992), cellulose acetate (Fonong, 1987), nylon net (Mascini *et al.*, 1987) and an immuno-affinity membrane called Biodyne (Bardeletti *et al.*, 1986). Lactate oxidase has also been successfully entrapped in an electro-polymerised o-phenylenediamine film, formed on a platinum electrode *in situ* in a flow injection system, and was used to determine lactate concentrations in serum (Dempsey *et al.*, 1993; Palmisano *et al.*, 1994).

Continuous monitoring of subcutaneous lactate levels has been achieved by using either a suction effluent technique (Ito *et al.*, 1995), or microdialysis (Volpe *et al.*, 1995) in conjunction with a lactate oxidase membrane and platinum electrode in a flow injection system.

Miniature enzyme electrodes comprising of a platinum wire within a stainless steel needle, coated with oxidase enzyme and membranes, have been fabricated with the intention of continuously monitoring glucose or lactate *in vivo*, although surprisingly few *in vivo* lactate measurements have been reported (Battersby & Vadgama, 1988; Churchouse *et al.*, 1986; Hu *et al.*, 1993; Shichiri *et al.*, 1982). Recently, a similar needle type L-lactate sensor was applied to food analysis (Kim *et al.*, 1996).

Miniature, integrated thin-film biosensors have also been developed by Urban *et al.* (1994; 1994a) to detect glucose, lactate and the pH of test solutions. The sensors were produced on a flexible, planar polyimide base using thin photo-patternable enzyme membranes over evaporated titanium-platinum tracks that acted as electrodes. Polyimide was also used as a flexible base for a three electrode chip for either flow injection analysis or direct assay with the intention of *in vivo* cardiovascular monitoring of lactate by Marzouk *et al.* (1996). Microelectronically fabricated gold strips were electrochemically coated with platinum and then three membrane layers were applied to the working electrode to entrap lactate oxidase, exclude interfering compounds and extend the detection range.

Probes made from platinum electrodes coated with lactate oxidase immobilised or entrapped within membranes, to detect hydrogen peroxide, have also been used for the non invasive determination of lactate within sweat and saliva (Faridnia *et al.*, 1993; Guilbault *et al.*, 1995; Palleschi *et al.*, 1991; Palleschi *et al.*, 1993).

Amperometric enzyme electrodes have also been prepared by entrapping lactate oxidase over carbon electrodes. Mizutani *et al.* (1993 and 1995) employed glassy carbon electrodes at around 1 V (Ag/AgCl) to detect the enzymically generated hydrogen peroxide. Interference from oxidisable species such as ascorbic acid, uric acid and acetaminophen can be problematic at these potentials. Mizutani *et al.* (1995) achieved lower interference by using a polyion complex membrane to immobilise the lactate oxidase. Other attempts to lower interference at carbon electrodes include modification of the surface or using artificial electron acceptors (mediators, Section 1.7.4.3.), to lower the potential of the working electrode.

Modification of carbon electrodes using transition metals for the detection of hydrogen peroxide at low operating potentials (around +350 to +450 mV vs. Ag/AgCl) has been used in combination with lactate oxidase to produce sensors that selectively detect L-lactate. Scheller *et al.* (1986) sputtered a carbon rod with palladium and gold and used this catalytic electrode with a lactate oxidase membrane in a flow injection system to detect lactate concentrations of spiked buffer solutions. They recorded a low sensitivity to ascorbic acid in comparison to lactate which was not possible at solid platinum electrodes. Platinum was electrochemically deposited onto carbon rods by Hajizaheh *et al.* (1991), onto which poly(vinyl alcohol) and lactate oxidase layers were cross-linked using gamma radiation to make a sensor for lactate. Lactate oxidase was found to be active in the presence of the polymer under radiation doses as high as 40 Mrad, demonstrating that lactate oxidase can be employed in sensors that need to be sterilised for invasive monitoring. Platinised carbon has also been electrodeposited onto screen-printed carbon electrodes by Hart *et al.* (1996). A screen printed layer of lactate oxidase was retained on the surface by a cellulose acetate membrane, applied in some cases by ink-jet printing. Hart's group later screen printed the entire sensor using a platinised carbon ink under the lactate oxidase layer and were able to estimate the lactate concentration in diluted samples of yoghurt and buttermilk (Collier *et*

al., 1996). The use of automated printing technology to fabricate large numbers of sensors allows the sensors to be treated as disposable since the unit cost is reduced, an advantage over hand-fabricated devices designed for clinical monitoring.

Palladium-modified graphite powder has been used in conjunction with a sol-gel and lactate oxidase to produce lactate biosensors with a renewable, controlled reactive layer (Sampath & Lev, 1996). Sampath and Lev concluded that the palladium-modified, carbon-ceramic biosensors could be used as either moulded or thick-film sensors with excellent stability and repeatability.

Rhodinised carbon electrodes used with lactate oxidase have recently been described by White *et al.* (1994a) and Wang's group (Wang & Chen, 1994a; Wang *et al.*, 1995a). Lactate sensors were constructed by White *et al.* (1994a) using lactate oxidase immobilised in hydroxyethyl cellulose on the surface of graphite rods electroplated with rhodium, and overlaid with a cellulose acetate membrane. The sensors, integrated into a flow injection system, accurately detected the enzymically generated hydrogen peroxide in buffer and mammalian cell culture solutions at +400 mV (Ag/AgCl). A combination of thick film printing and laser machining techniques were employed to construct enzyme microelectrode arrays by Wang *et al.* (1994a). These disposable microdisc arrays of carbon were highly reproducible and served as hosts for the electrochemical co-deposition of lactate oxidase and rhodium. Wang *et al.* (1995a) later reported the use of rhodinised carbon and lactate oxidase as a carbon paste electrode in a flow system to selectively detect lactate at 0 V (Ag/AgCl).

1.7.4.3. Lactate Oxidase and Mediators.

There have been a number of reports of mediators used at carbon electrodes in conjunction with lactate oxidase, including ferrocene and its derivatives (Boujtita *et al.*, 1996; Kulys *et al.*, 1992a; Preneta, 1987; White *et al.*, 1992), hexacyanoferrate (ferro-/ferri- cyanide) (Shimojo *et al.*, 1993; Uenoyama *et al.*, 1993), octacyanotungstate and octacyanomolybdate (Taniguchi *et al.*, 1988), Meldola Blue (Kulys *et al.*, 1992a), Methylene Green (Kulys *et al.*, 1993), tetrathiafulvalene with tetracyanoquinodimethane (TTF/TCNQ) (Nguyen & Luong, 1993) and tetrathiafulvalene alone (Liu *et al.*, 1995; Mulchandani *et al.*, 1995; Palleschi &

Turner, 1990; White *et al.*, 1992; Zhao & Luong, 1993). Surprisingly, only Uenoyama's group report of mass fabricated devices that could be used for the measurement of lactate in whole human blood (Shimojo *et al.*, 1993), although Kulys *et al.* (1993) used their hand-fabricated methylene green and carbon-paste electrodes for L-lactate determination in goat whole blood.

1.7.4.4. Lactate Oxidase Co-immobilised with Other Enzymes.

Lactate dehydrogenase has been used to regenerate lactate from the pyruvate formed by lactate oxidase to amplify the response to L-lactate; both enzymes were co-immobilised over an oxygen electrode (Mizutani *et al.*, 1984; Mizutani *et al.*, 1985). These two enzymes have also been coupled with the mediator tetrathiafulvalene to assay for NADH, L-lactate and pyruvate (Zhang *et al.*, 1996). The compounds were immobilised on a glassy carbon electrode, poised at +200 mV (SCE), and an amplified response was detected due to recycling of L-lactate and pyruvate.

Carbon paste electrodes modified with lactate oxidase and horseradish peroxidase have been reported (Spohn *et al.*, 1996a; Spohn *et al.*, 1996b). The electrodes were able to detect L-lactate at low potentials (between -50 and +50 mV vs. Ag/AgCl) due to the adsorbed or covalently bound horseradish peroxidase on the carbon particles enabling direct detection of hydrogen peroxide. Horseradish peroxidase has also been linked with an osmium poly(vinylpyridine) redox polymer to directly measure hydrogen peroxide concentrations generated by oxidase enzymes, including lactate oxidase (Yang *et al.*, 1995). There have also been several reports describing the use of osmium poly(vinylpyridine) compounds to non-diffusionally relay electrons from lactate oxidase to carbon electrodes to detect L-lactate directly (Katakis & Heller, 1992; Kenausis *et al.*, 1996; Wang & Heller, 1993; Wang *et al.*, 1992a). This lead to the fabrication of miniature flexible amperometric probes for L-lactate determination by Wang and Heller's group (1993 and 1992a) with the intention of applying the probe to continuous *in vivo* L-lactate monitoring, although no reports of this have yet been published.

Lactate oxidase has been successfully applied to the fabrication of a wide range of amperometric lactate devices, allowing rapid determinations without the constraints imposed

by lactate dehydrogenase or flavocytochrome b_2, that is, addition of cofactors and electrode fouling by reaction products. The reported flexibility and reliability of lactate oxidase in a diverse range of sensor chemistries therefore promotes itself for use in this work.

1.8. Aim of this Work.

The aim of this thesis was to develop a mass-producible amperometric enzyme electrode employing lactate oxidase for the detection of L-lactate in undiluted, whole blood. Starting from relatively simple carbon electrodes, sensors will evolve into mass-producible enzyme electrodes, selective for L-lactate. A range of chemically modified electrodes combined with lactate oxidase are to be first investigated in buffer solutions. The most suitable enzyme electrode will then be selected and developed into a mass-producible sensor to measure L-lactate in biological fluids. This is to be aided by the addition of membranes to further enhance the detection range and selectivity. The ultimate aim is to provide a sensitive and selective measurement of blood lactate simply and inexpensively, leading to possible development of an *in vivo* device.

CHAPTER 2:

CHEMICALLY MODIFIED CARBON ELECTRODES

2.1. INTRODUCTION.

This chapter describes the characterisation and construction of sensors for L-lactate detection using different electrode modifications. Where appropriate, the screen printing technique was used, facilitating the transfer to a mass-producible process and enabling highly reproducible, inexpensive devices to be rapidly manufactured. The specific aims are detailed in Section 2.1.7. Hydrogen peroxide generated by oxidase enzyme reactions can be electrocatalytically oxidised (or reduced) at modified carbon electrodes (Gorton, 1985; Karyakin *et al.*, 1994a; Wang *et al.*, 1995b). This chapter describes experiments carried out on three types of such modified electrodes:-

- hexacyanoferrate (III) films;
- Prussian Blue (iron hexacyanoferrate);
- rhodium on carbon.

Mediation of the enzyme reaction by tetrathiafulvalene was also studied and applied to screen printed electrodes. Polypyrrole was used as an immobilisation matrix for lactate oxidase and possible direct electron transfer was measured. The chemistries were evaluated for sensitivity and selectivity for L-lactate and metallised carbons were chosen for further study.

2.1.1. Screen Printing.

Screen printing is a form of graphic reproduction where a quantity of ink or other viscous compound is deposited as a film in a controlled manner to form a repeatable pattern of certain thickness (Alvarez-Icaza & Bilitewski, 1993; Goldberg *et al.*, 1994). This entails squeezing ink through a gauze (or mesh) on to a surface. There are five components needed for making a screen print (see Figure 2.1.):-

- A frame over which a gauze is stretched to form the screen.
- A photo stencil applied to the gauze of the desired pattern.
- A secure platform on which to place the substrate and frame.
- A squeegee - a flexible yet resilient blade fixed into a holder.
- An ink in the form of a paste or viscous compound.

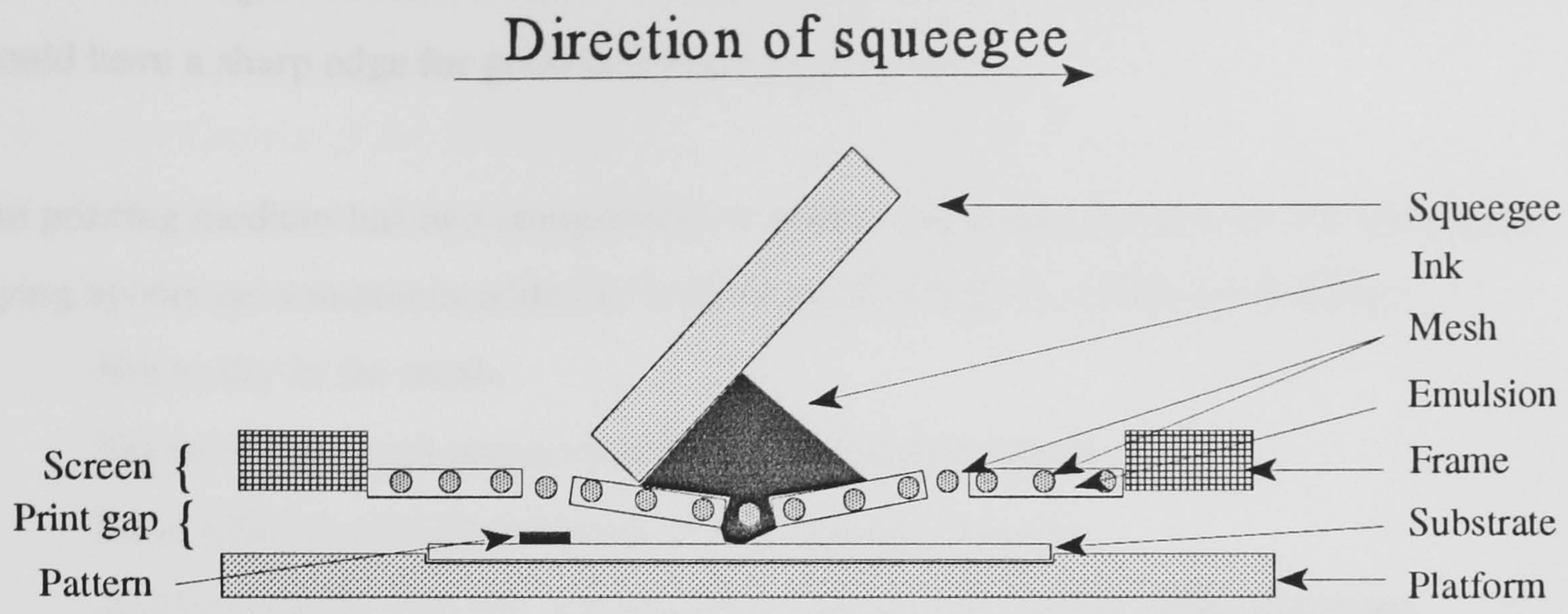


Figure 2.1. Screen printing process.

These components can be grouped together and automated by a screen printer (e.g. DEK Printing Machines Ltd., DEK-ALBANY, Weymouth, Dorset) to enable the screen printing process to become less labour intensive, and highly repeatable.

The screen is attached to a metal frame; wood and plastic tend to warp under the squeegee pressure and cleaning processes. The mesh of the screen can be made of Nylon, poly(ester) or stainless steel. The minimum definition of line that can be printed is usually determined by three times the mesh thread diameter. The distance between the threads of the mesh is usually less than three times the particle size of the ink. An ultra-violet light-sensitive poly(vinyl) chloride emulsion is used to create the print pattern on the screen mesh.

The squeegee is an important part of the screen printing process. The squeegee brings the screen into intimate contact with the substrate and the rate of release of the screen is controlled by regulating the squeegee pressure and speed. A high speed pass of the squeegee results in a thick deposit of ink and high pressure results in a thin deposit of ink. The distance between the screen and the substrate (print gap) can be gauged using the width of the screen multiplied by: 0.004 for stainless steel mesh, 0.006 for poly(ester), or 0.01 for Nylon, as a guideline. The squeegee forces the ink into the openings of the screen and also shears and removes excess ink as it passes. Ideally, the squeegee should be pulled across the

screen at an angle between 45–60°. The squeegee is usually made of poly(urethane) and should have a sharp edge for good definition of the pattern.

The printing medium has two components; a carrier and an active element but retarder or drying agents are sometimes added as well. Important features of the ink include:-

- Not to dry in the mesh.
- Not to require high pressure to force it through the mesh.
- Easy adherence to the substrate as the squeegee passes.
- To flow readily over the screen on the non-printing stroke of the squeegee.

The rheology of the ink is therefore very important and there are manufactures who specialise in forming ideal printable inks (e.g. Gwent Electronic Materials, Gwent, Wales).

2.1.2. Modified Carbon Electrodes.

The biosensor transducer should include the following features (Lowe, 1985):-

- High specificity for the analyte and response in the relevant concentration range.
- Fast response time (typically 1-60 s).
- Amenable to miniaturisation and practically suitable.
- Reliable; it should not be adversely affected by environmental changes like temperature.

Carbon has the above features and it is electrochemically attractive for sensing applications because it has a wide anodic potential range, low electrical resistance with a low background current and is relatively inexpensive (Gilmartin & Hart, 1995; Wang *et al.*, 1996). Carbon based printing inks offer the advantages of carbon but are constrained by the printing process to offer a low carbon content and a complex surface once printed. This compromises the analytical performance and redox activity (Wang *et al.*, 1996) and carbon alone often requires a high activation overpotential to oxidise or reduce biomolecules (Frew & Hill, 1988; Wring, 1992). However, by deliberately modifying the surface of the carbon particles with chemical and biological components the selectivity and sensitivity of the sensor can be enhanced (Gorton, 1985; Wang, 1991). Chemical modification can facilitate the charge transfer process between the electrode and biological component and can significantly reduce the overpotential by providing electrocatalytic effects (Wring, 1992). For selective amperometric detection, the optimum operating potential should be in the range -100 to 0 mV (SCE) since most common

interfering compounds for biological applications (e.g., ascorbic acid, uric acid, bilirubin, paracetamol, catecholamines and dissolved oxygen) are neither oxidised or reduced at these potentials (Gorton *et al.*, 1991). For most electrode materials the sensitivity and low noise characteristics will also be enhanced because the background current changes from positive to negative (or *vice versa*) and is therefore relatively low in this potential region (Gorton *et al.*, 1991).

2.1.3. Modification with Hexacyanoferrate.

There have been reports of hexacyanoferrate films being formed on carbon fibres to produce miniaturised glucose biosensors with a potential for *in vivo* sensing (Jaffari & Pickup, 1996; Jaffari & Turner, 1997). These films are thought to be a Prussian Blue analogue (Jaffari & Turner, 1997) and this thesis details experiments carried out using the film in conjunction with lactate oxidase to form lactate sensors. Jaffari's work was taken further in this thesis by electrochemically forming hexacyanoferrate films on screen-printed electrodes with the aim of mass fabricating L-lactate sensors.

It has been reported by Itaya (1984) that Prussian Blue (iron hexacyanoferrate) on glassy carbon electrodes can electrocatalytically reduce hydrogen peroxide, commencing at about 0.5 V (SCE). The Prussian Blue was electrochemically formed as a film on the carbon surface. A UK patent was issued to Turner and Jaffari in 1994, detailing work of an electro-catalytic film formed with hexacyanoferrate (III) on carbon electrodes, and later an international patent was issued (Jaffari & Turner, 1994; Turner & Jaffari, 1995). Karyakin (1994a) also deposited Prussian Blue as a film on glassy carbon electrodes but then deposited glucose oxidase on the surface to produce glucose sensors which detected hydrogen peroxide enzymically produced from glucose at +180 mV (Ag/AgCl/KCl 1M). The same detection principle was used in this work, Prussian Blue-modified carbon was printed onto electrodes with the aim of electrocatalytically detecting hydrogen peroxide. Prussian Blue is commercially available and can be mixed directly with a carbon ink and printed onto base electrodes.

2.1.4. Modification with Rhodium.

Highly dispersed noble metals bound with carbon present an electrocatalytic effect due to synergistic and electronic interactions (Mukerjee, 1990). Metallised carbon electrodes can operate at low potentials with preferential detection of hydrogen peroxide over other potential interferences commonly found in biological fluids. They also offer a fast response with high sensitivity and uncomplicated manufacture (Wang *et al.*, 1995b). Rhodium on carbon (rhodinised carbon) was seen as an interesting electrocatalytic material and data published during this work shows it to be more sensitive and selective towards hydrogen peroxide than platinised carbon (White *et al.*, 1994b), the most commonly used metallised carbon in biosensors. Rhodinised carbon is commercially available as a powder and amenable to the screen printing process. There are relatively few reports of rhodinised carbon used in lactate sensors (Wang & Chen, 1994a; Wang *et al.*, 1995a; White, 1993) and all are manufactured by electrodeposition techniques which is more labourious than screen printing.

2.1.5. Artificial Electron Transfer.

There are two main approaches for linking redox enzymes directly to an electrode (Cardosi & Turner, 1987; Frew & Hill, 1988):-

a) indirect and mediated electron transfer;

b) direct and unmediated electron transfer.

However, a protective protein and glycoprotein shell surrounds the active centre of redox enzymes and has two important functions (Heller, 1990):-

a) it stabilises the enzyme structure,

b) it prevents indiscriminate electron exchange between natural redox macromolecules.

The redox centre of lactate oxidase, flavine adenine dinucleotide (FAD) is deep within the enzyme molecule. When lactate oxidase converts lactate to pyruvate, the reduced form of FAD (that is, $FADH_2$) combines oxygen, the natural electron acceptor, with hydrogen to form hydrogen peroxide. These substrates and products of the enzyme reaction can easily diffuse towards and away from the redox centre and it is the aim of an artificial mediator to access the redox centre just as freely. An artificial mediator must also be reduced more rapidly than oxygen and be electroactive at low potentials in order to be successful in an amperometric sensing device (Cardosi & Turner, 1987). This method of indirect and mediated electron

transfer largely overcomes the oxygen dependence of amperometric oxidase sensors based on substrate or product detection.

Tetrathiafulvalene was used as a mediator in this study for several reasons. It requires a low potential to generate the active species, thus minimising interference and has a low solubility, confining it to the electrode surface. Although ferrocene (and derivatives) have these advantages (Cardosi & Turner, 1987a) it has been found that tetrathiafulvalene gives a larger response with lactate oxidase than 1,2-dimethyl ferrocene (White *et al.*, 1992). It has previously been reported that the ferrocene carboxylic acid is an excellent mediator for glucose oxidase but is not as good a mediator for lactate oxidase and yet another mediator (octacyanomolybdate ($Mo\{CN\}_8^{4-}$)) was good for lactate oxidase and yet poor for glucose oxidase (Taniguchi *et al.*, 1988). This indicates that the interaction between mediator and enzyme is quite specific to the individual species involved.

2.1.6. Direct Electron Transfer.

Direct and unmediated electron transfer is more challenging to implement than employing a mediator compound. For a redox enzyme to communicate with an electrode directly, the distance of over which the electron travels must be less than 3Å, a greater distance results in an exponential decay of the rate of electron transfer (Heller, 1990). This often requires the outer, protective, shell of the redox enzyme to be taken away, but then maintaining its stability becomes a problem (Alvarez-Icaza & Schmid, 1994). The electrode surface can be modified so that a suitable interface for interaction with the protein is provided (Frew & Hill, 1988). Unlike a mediator, a modifier compound is electrochemically inactive in the potential range of the electron transfer observed for the enzyme (Cardosi & Turner, 1987; Frew & Hill, 1987).

There have been several recent reports claiming direct electron transfer between glucose oxidase and polypyrrole modified electrodes (Koopal, 1992; Koopal *et al.*, 1994; Koopal *et al.*, 1992a; Koopal *et al.*, 1992b). Glucose oxidase was irreversibly adsorbed onto polyprrole which was formed within microtubules of cyclopore or nucleopore membranes, or pores of a latex matrix. One side of the membrane was sputtered with platinum which was made to contact a carbon electrode. The latex matrix was formed on a sputtered platinum surface and

then removed and sieved to produce a powder which was incorporated into a conducting carbon ink and printed onto carbon electrodes.

One school of thought is that the glucose oxidase communicated directly with the electrode (Koopal, 1992; Koopal *et al.*, 1994; Koopal *et al.*, 1992a; Koopal *et al.*, 1992b). It was thought that due to the intimate contact between the active site of glucose oxidase and the corrugated surface of polyprrole, direct electron transfer occurred. This entailed the flavin adenine dinucleotide (FAD) of glucose oxidase passing electrons directly to the conducting polyprrole and thus to the carbon electrode.

Other researchers dispute the validity of these mechanisms, claiming that a polyprrole matrix immobilised the glucose oxidase but it was the glucose or the enzymically produced hydrogen peroxide which was detected at the platinised carbon surface (Belanger *et al.*, 1989; Kuwabata & Martin, 1994). There are reports of the direct oxidation of glucose at platinised carbon electrodes (White *et al.*, 1992; White *et al.*, 1994b) and it is also known that a platinised carbon surface will oxidise hydrogen peroxide at the potentials used in these studies (Gunasingham & Tan, 1989b; Tay *et al.*, 1988).

There have been no publications of the use of lactate oxidase with polyprrole in this format and it was hoped that by using a different enzyme it could be determined whether or not polyprrole communicates directly with the enzyme. This new format may also provide a viable alternative to other detection methods.

2.1.7. Aims.

The aims of this chapter were to:-

- explore traditional (carbon modified with rhodium or tetrathiafulvalene) and novel (carbon modified with hexacyanoferrate films, Prussian Blue or polypyrrole) amperometric transducer chemistries for electrocatalytic behaviour with hydrogen peroxide and/or L-lactate;
- apply these transducer chemistries to the fabrication of screen printed electrodes;
- evaluate the transducers for application to an L-lactate amperometric enzyme electrode.

2.2. EXPERIMENTAL.

2.2.1. General Reagents.

Reagents of analytical grade from BDH Limited (Poole, Dorset), Aldrich Chemical Company (Gillingham, Dorset) and Sigma Chemical Company (London) were used unless otherwise stated. Water purified by reverse osmosis (an Elgastat system provided by The Elga Group, Buckinghamshire) was always used. The buffer used was phosphate buffered saline (pH 7.2, 0.1 M sodium phosphate and 0.15 M sodium chloride). Lactate oxidase (*Pediococcus Species*, E.C.1.1.3.2.) was purchased from Genzyme with an activity of 35 $U.mg^{-1}$.

2.2.2. Carbon Rod Electrode Fabrication.

Spectroscopic graphite rods (6 mm diameter, Ringsdorf-Werke GmbH, Germany) were sawn into approximately 10 mm lengths and sealed into plastic pipette tips (cut to the correct size) using epoxy resin adhesive (Araldite, Ciba-Geigy) (see Figure 2.2.). Care was taken to ensure that the seal was water-tight and that the rod was flush with the end of the tip. A length of insulated wire was connected to the carbon rod using silver loaded epoxy adhesive (RS Components, Corby, Northamptonshire) and kilned at 115°C for 30 minutes and then 60°C for 90 minutes, to ensure a good contact. The electrodes were smoothed using silicon carbide abrasive paper (400 and 1200 grade, RS Components, Corby, Northamptonshire) and then polished with decreasing particle sizes of aluminium oxide and diamond paste until a flat surface was achieved with 0.3 μm aluminium oxide (highly pure for polishing from BDH, Poole, Dorset). The electrodes were sonicated in acetone and water to remove particulates and grease.

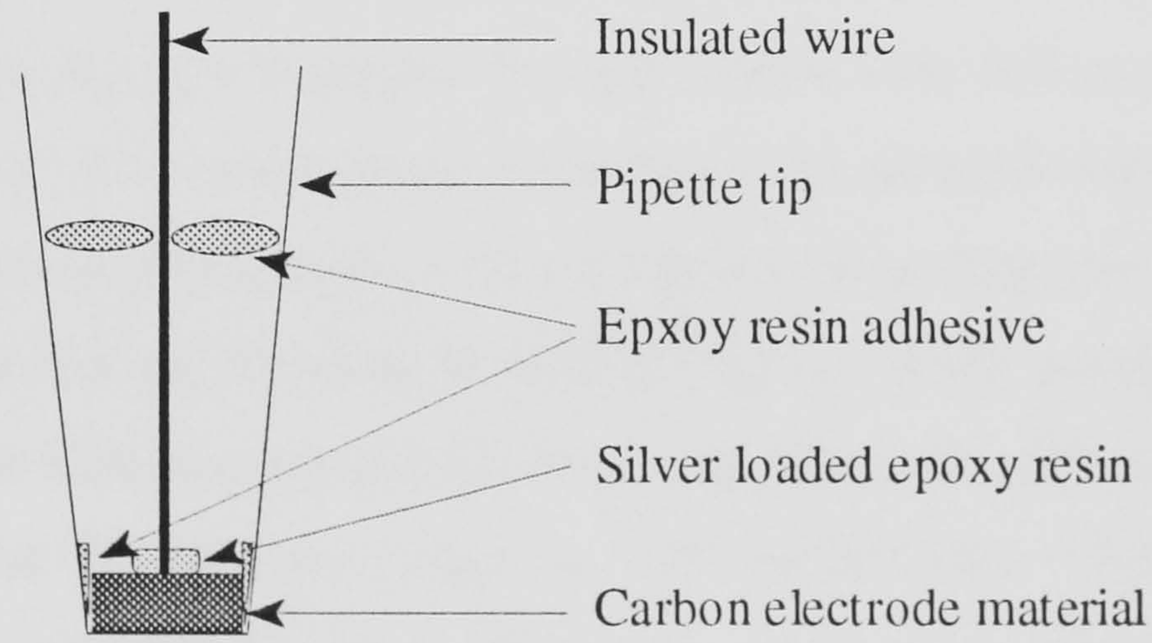


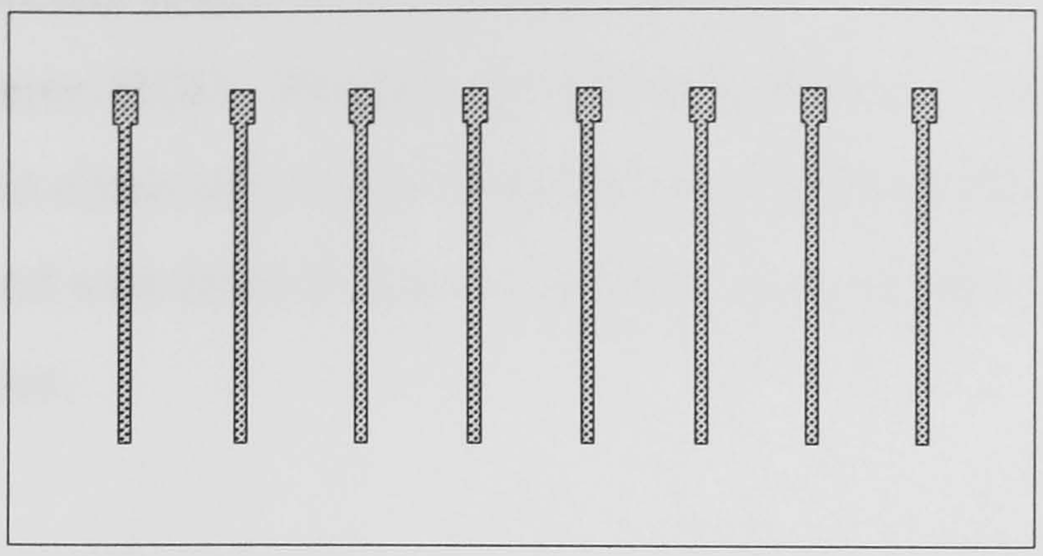
Figure 2.2. Typical hand fabricated electrode fabrication.

2.2.3. Carbon foil Electrode Fabrication.

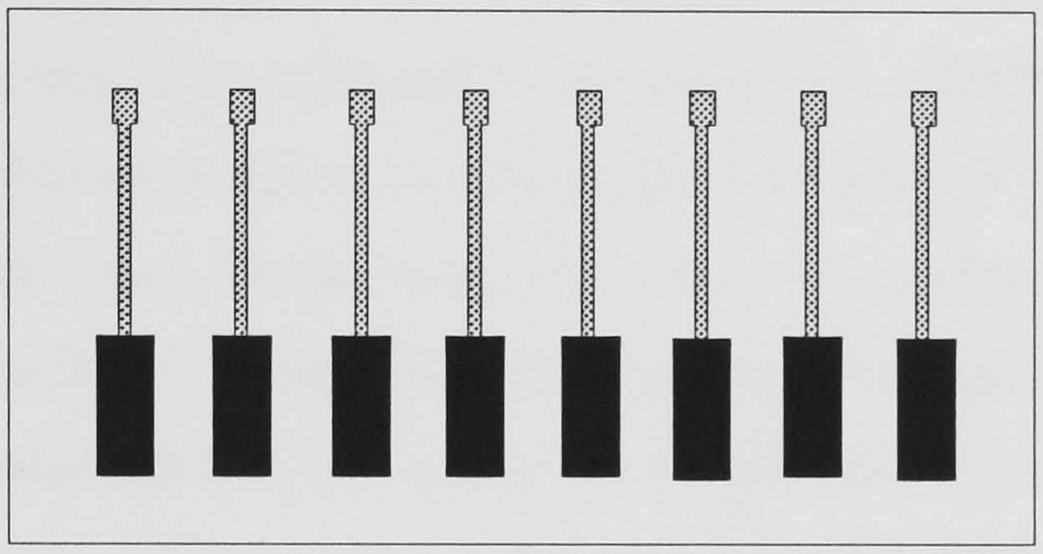
Electrodes were constructed as described by Cass *et al.* (1984). Graphite foil (1 mm thick from Le Carbonne, Portslade, Sussex) was cut into 9 mm diameter discs using a cork borer. An insulated wire was connected to the disc using silver loaded epoxy adhesive (RS Components, Corby, Northamptonshire) and placed in an oven at 115°C for 30 minutes to make highly conductive. The graphite discs were then sealed into a plastic pipette tip using epoxy resin adhesive (Araldite, Ciba-Geigy) to ensure that the disc edges were not exposed (see Figure 2.2.). The electrodes were then dried in a 60°C oven for between 10 and 20 hours. Polishing of the electrodes was carried out using 0.3 μm aluminium oxide (highly pure for polishing from BDH, Poole, Dorset) and the surface was cleaned by sonicating in acetone and water.

2.2.4. Screen-Printed Electrode Construction.

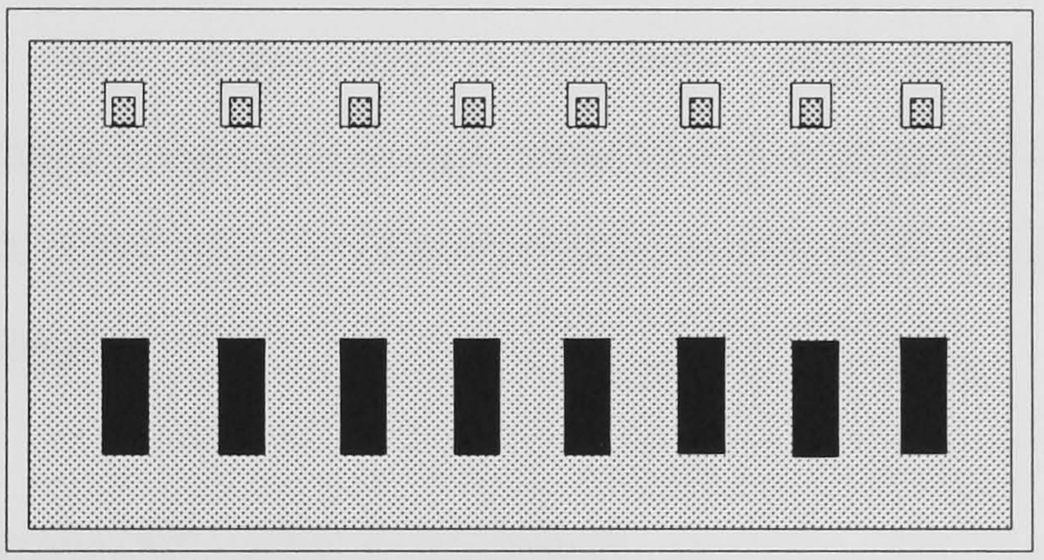
A high performance multi-purpose precision screen printer DEK 245 (DEK, Weymouth) was used to manufacture planar arrays of eight electrodes in several stages. The substrate used was either poly(vinyl) chloride (Genotherm, Sericol, Surrey, UK) or transparency film for plain paper copiers (PP 2500, 3M, France) onto which a sequence of patterns was printed (see Figure 2.3.). All screens employed a poly(ester) mesh with a 45° orientation surrounded by a metal frame. The silver tracks were printed using a conducting silver-based flexible polymer thick-film ink (Electrodag 477 SS RFU, Acheson Colloids Company, Plymouth, UK) through a screen pattern with 200 counts/inch and an 18 μ emulsion thickness. The ink was allowed to dry for 180 minutes at 40°C. A carbon pad was printed onto the end of the silver track using a screen with 390 counts/inch and 18 μ emulsion thickness and a graphite-based polymer thick film ink (Electrodag 423 SS, Acheson Colloids Company, Plymouth, UK). This was dried at 40°C for 60 minutes. An insulation shroud was applied through a screen with 390 counts/inch and an emulsion thickness of 25 μ. One of two types of insulation shroud were employed; a highly resistant polymer which needed to be heated in order to harden (Blue insulation fired at 120°C for one hour, 242-SB, ESL Europe, Reading, Berkshire, UK) or a quick drying screen printing ink with high resistance to water penetration (Black or Light Green insulation shroud dried at room temperature for 30 minutes, Glanz Jet HG 40/NT-Neu, Wiederhold Siebdruckfarben, Coates Brothers GmbH, Nurnberg, Germany). All inks were diluted with



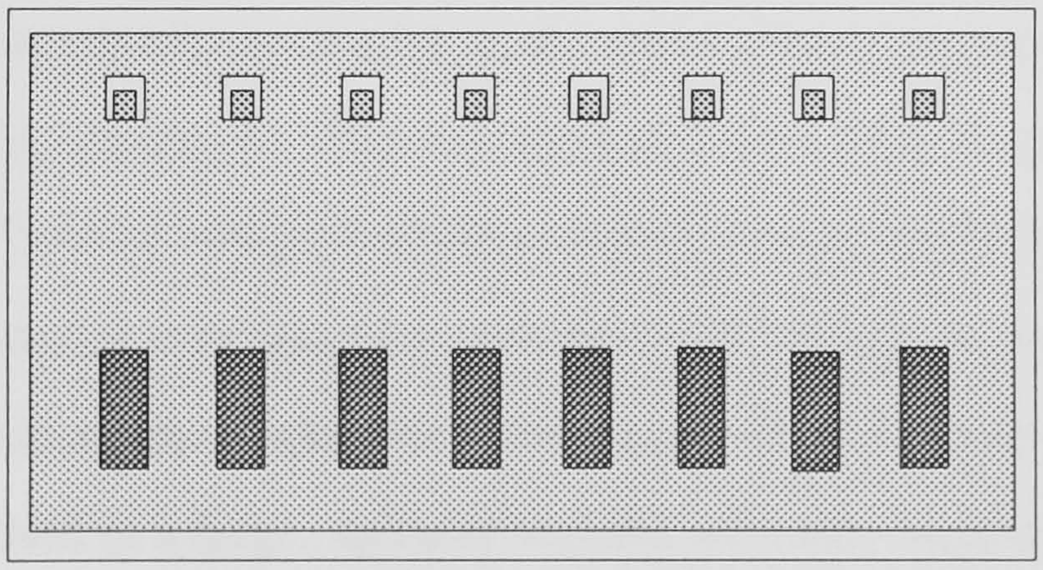
1. Silver tracks for eight electrodes.



2. Silver track plus carbon pads.



3. Insulation shroud covering electrodes.



4. Working electrodes printed on top of carbon pads and silver tracks.

Figure 2.3. Sequence for screen printing electrodes.

either 2-butoxyethyl acetate (BEA, 99%, Aldrich Chemical Company, Gillingham, Dorset) or retarder (Verzogerer HGD, Wiederhold Siebdruckfarben, Coates Brothers GmbH, Nurnberg, Germany) to obtain the correct consistency for screen printing. The screens were cleaned in a fume hood with BEA as soon as printing was complete, following health and safety codes of practice.

All commercial carbon screen printing inks contain additional proprietary materials to aid the printing and drying process and are not ideal for electrochemical experimentation. Consequently carbon inks were produced in the laboratory specifically for the working electrode. A carbon ink was made by combining graphite particles (T15 carbon graphite, Lonza G&T Limited, Sins, Switzerland) with either an aqueous or organic binder. Hydroxyethyl cellulose (HEC, Aldrich Chemical Company, Gillingham, Dorset) was made into a solution with buffer (2% w/v) and mixed in a weight ratio of 1:3 with graphite for 15 minutes. This was printed using a water-resistant screen with 200 counts/inch and 23 μ emulsion thickness. Alternatively, cellulose acetate (acetate content ~ 40%, Sigma Chemical Company, London) was made into a solution (4% w/v in 1:1 solution of acetone:cyclohexanone), mixed in a weight ratio of 1:3 with graphite for 15 minutes and printed quickly (the ink dried fast). A solvent resistant screen with the same design of water-resistant screen was used.

2.2.5. Hexacyanoferrate (III)-Film Formation.

This procedure was taken from work carried out by Jaffari (1994). Either a graphite rod or screen-printed carbon electrode (carbon ink made with cellulose acetate as described above) was used as the working electrode. This was cycled fifteen times from 0 to 2.5 V (SCE) at a scan rate of 0.2 $V.s^{-1}$ in 5 or 10 ml of stirred potassium hexacyanoferrate (III) solution (0.1 M in water). A new solution was taken for each electrode and the stock solution, made fresh each day, was stored at room temperature in the dark. Once the procedure was complete, the electrode was washed in a large, agitated volume of water until the water ran clear. All solutions of hexacyanoferrate were disposed of via a waste aqueous solution bottle, and health and safety codes of practice were followed throughout.

2.2.6. Carbodiimide Immobilisation of Lactate Oxidase.

Lactate oxidase was immobilised on hexacyanoferrate film-modified carbon electrode using the method described by White (1993). A sodium acetate buffer (0.2 M, pH 5.5) was used to make a 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide solution (0.15 M) into which the carbon rod electrodes were immersed for 1¾ hours. The solution was stirred continuously. The carbodiimide electrodes were then washed and sonicated in water and then dried. A 5 µl solution of lactate oxidase (200 $mg.ml^{-1}$ in 0.02 M potassium phosphate buffer, pH 7.0) was deposited onto the surface of an electrode held vertically and left to dry for 90 minutes under atmospheric conditions (ambient temperature and pressure). The enzyme electrodes were then stored at 4°C in the dark. Before testing the enzyme electrodes, they were washed thoroughly in buffer to remove unbound lactate oxidase.

2.2.7. Prussian Blue Ink Formation.

Cellulose acetate based carbon ink was mixed with Prussian Blue (PB, iron (III) hexacyanoferrate, Aldrich Chemical Company, Gillingham, Dorset) in a weight ratio of 1:1 or 1:5, PB:carbon. This was then printed onto base electrodes using the solvent resistant working electrode screen (200 counts/inch and 23 μ emulsion thickness).

2.2.8. Rhodinised-Carbon Electrode Manufacture.

Base electrode arrays were screen printed as described above. Rhodium on carbon (5% metal on carbon, Avocado Research Chemicals Limited, Lancaster) was mixed with graphite powder and hydroxyethyl cellulose solution (2% w/v buffer) in a weight ratio of 1:1:3. The water-resistant working electrode screen was used to print the ink onto the array and allowed to dry either at room temperature (for one hour) or at 40°C for 40 minutes. The ink, even once dry, was unstable in buffer (it broke away from the carbon overlay pad) and so a retaining membrane of cellulose acetate (2% w/v in acetone) was applied by dip-coating and allowed to dry for one hour before testing took place. Electrodes were stored in the dark at room temperature before use and disposed of once tested.

2.2.9. Lactate Oxidase Ink Formation.

Carbon graphite (100 mg of T15, Lonza G&T Limited, Sins, Switzerland) was mixed with HEC (150 mg of 2% w/v in buffer) and then lactate oxidase (10 mg LOD, Genzyme) was added. The ink was mixed thoroughly for approximately 15 minutes and then printed onto the rhodinised-carbon screen-printed electrodes, using the water-resistant working electrode screen. A retaining membrane of cellulose acetate (2% w/v in acetone) was dip coated onto the electrodes and dried for 1 hour at room temperature before testing. All enzyme electrodes were stored at 4°C with silica gel.

2.2.10. Tetrathiafulvalene Modification.

The procedure followed was that of Palleschi and Turner (1990). Graphite foil electrodes were immersed in a tetrathiafulvalene solution (1% w/v in acetone) for 2 hours in a sealed vessel and then dried at ambient temperature for one hour. Tetrathiafulvalene was stored and used under a nitrogen atmosphere in a fume hood whenever possible due to its ease of oxidation in air. Health and safety codes of practice were followed. Alternative methods using a tetrathiafulvalene solution (1% w/v in acetone) were also tested. A known volume was added to an electrode surface, the solvent drying to leave the tetrathiafulvalene adsorbed or absorbed on or in the carbon material. Carbon powder (graphite T15, Graphites and Technologies, Sins, Switzerland) was mixed with a tetrathiafulvalene solution (2.5% w/v in diethyl ether), dried, and then lactate oxidase and hydroxyethyl cellulose (2% w/v in buffer) were added in varying proportions to make an ink. This was printed onto base electrodes using the water-resistant working electrode screen.

Screen-printed electrodes with a water based carbon working electrode were baked to remove any moisture at 60°C for at least 24 hours. Tetrathiafulvalene was applied to the surface with a pipette (6 x 5 μl, 1% w/v in acetone) and dried in a stream of nitrogen before lactate oxidase was adsorbed onto the surface.

2.2.11. Lactate Oxidase Adsorption onto Modified Carbon Electrodes.

Modified carbon-foil electrodes were placed in a lactate oxidase solution (25 $mg.ml^{-1}$ in buffer) for at least 24 hours before testing, to allow the enzyme to adsorb onto the carbon. The electrodes were stored in the enzyme solution at 4°C.

Alternatively, to achieve better reproducibility, an accurate volume of lactate oxidase solution (2 x 10 μl, 25 $mg.ml^{-1}$ in buffer) was applied to the carbon surface (foil or graphite) with a pipette and dried at ambient temperature before storing at 4°C with silica gel.

2.2.12. Lactate Oxidase Entrapment within Polypyrrole Coated Track-etch Membrane.

Track-etch membranes modified with polypyrrole were kindly supplied by Dr. C. Koopal. Polypyrrole was formed within the pores of Cyclopore® membranes of various pore sizes between 1000 and 200 nm, by means of oxidative polymerisation reaction of pyrrole (0.6 M) and iron chloride (2 M) solutions (Koopal *et al.*, 1992b). The final pore size was determined by the polymerisation time and the original pore size. The membranes (as received) were bathed in buffer for 4 hours before immersing in a lactate oxidase solution (5 $mg.ml^{-1}$ LOD in buffer) on a rotary stirrer (150 r.p.m.) for 45 minutes at 4 °C. They were then placed in a desiccator, under vacuum, containing calcium chloride (anhydrous) for approximately 24 hrs at 4°C and then replaced in buffer and stored at 4 °C until use.

2.2.13. Enzyme Assay to Establish Activity of Lactate Oxidase.

A spectroscopic enzyme assay using horseradish peroxidase was used to determine the activity of lactate oxidase within the polypyrrole matrix on the track-etch membranes. Into each well of a 96-well microtitre plate was dispensed the following: 0.5 $mg.ml^{-1}$ tetramethyl benzidine dihydrochloride (TMB ImmunoPure® from Pierce, Illinois, USA), 100 µl; 60 $U.ml^{-1}$ horseradish peroxidase (HRP type II, 180 purpurogallin units.mg^{-1} solid from Sigma Chemical Company, London), 10 µl; and buffer or sample (as prepared below), 45 µl. After six minutes the reaction was terminated using 1 M sulphuric acid, 25 µl per well, and the absorbance determined at 450 nm, using the well containing buffer as a blank reference. Sections of track-etch membrane (approximately 25 mm^2) were immersed in a stirred 5 ml solution of lithium lactate (0.2 M in buffer). Three samples were taken before immersion

(time = 0) and then again at 1, 5 and 10 minutes. To obtain a calibration graph, known concentrations of hydrogen peroxide were used as sample in the enzyme assay. Track-etch membrane sample adsorbances could then be correlated to hydrogen peroxide concentrations. From the increase in hydrogen peroxide concentration, a guide to the enzyme activity was determined.

2.2.14. Flow Injection Analysis.

A flow cell was used which incorporated a glassy carbon working electrode, a silver/silver chloride reference and the carbon body made up the secondary electrode. A 1 mm thick Teflon spacer with a 0.27 cm^2 hole was inserted between the membrane and the secondary electrode, to insulate the active surface (see Figure 2.4.). The electrodes were linked to a computer-controlled electrochemical analyser (Autolab with GPES3 software, EcoChemie B.V., Utrecht, Holland) in chronoamperometry mode with an operating potential of +350 mV (Ag/AgCl). The flow cell was connected to a flow injection system comprising of a software driven key pad control device (Gilson, France) linked to two diluter syringe pumps

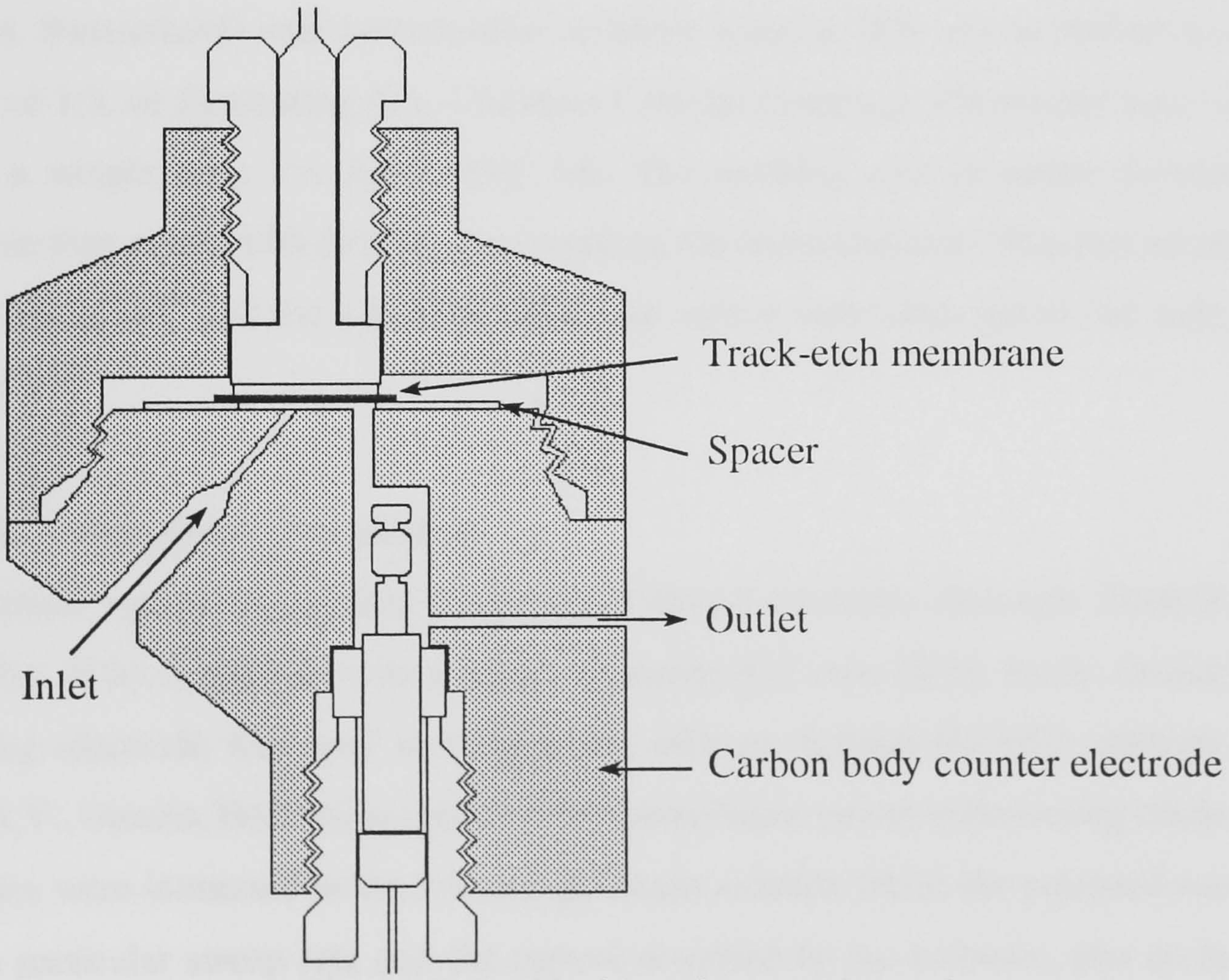


Figure 2.4. Cross section of flow cell.

(capacity of 5 ml, Model 401C, Gilson, France) and an injector valve actuator (6 ports, Model 817, Gilson, France). The flow rate, injection frequency and number of injections were set via the control pad; the conditions for track-etch membrane analysis were 0.5 $ml.min^{-1}$ flow rate, 10 injections with 10 s wait and 2 ml buffer between them. Phosphate buffered saline (0.1M phosphate, 0.15 M sodium chloride, pH 7.2) was used in all experiments.

2.2.15. Screen-Printed Electrodes Coated with Latex Beads Incorporating Lactate Oxidase Entrapped within Polypyrrole.

Dr. C. Koopal kindly supplied the latex beads incorporating lactate oxidase entrapped within polypyrrole (LPPLOD). Polypyrrole was deposited within a matrix of uniform latex particles by electrochemical oxidation in a buffered pyrrole solution (0.3 M in PBS) with a constant current applied (20 $mA.cm^2$) (Koopal *et al.*, 1992a) and lactate oxidase entrapped within the polypyrrole from a 5 $mg.ml^{-1}$ solution at 4°C for 4 hours and then dried overnight over calcium chloride in a desiccator. This was received as a powder with partical size less than 63 μm. The LPPLOD powder was mixed with carbon ink (Graphite T15, (Lonza G & T Limited, Sins, Switzerland) with hydroxyethyl cellulose solution (2% w/v in buffer) in a weight ratio of 1:3, or Electrodag 421, (Acheson Colloids Company, Plymouth) used as received) in a weight ratio 1:5, LPPLOD:C ink. The working area of screen printed electrodes were then coated with this ink, tape masking the unwanted area. This procedure was carried out at 4°C and the electrode dried and stored with silica gel at the same temperature.

2.2.16. Cyclic Voltammetry Procedures.

A three electrode system comprising a saturated Calomel reference electrode (Russell, Auchtermuchty, Fife), a platinum wire auxiliary electrode (0.5 mm, BDH, Poole, Dorset) and a working electrode was used in conjunction with an Autolab (GPES3 software, EcoChemie B.V., Utrecht, Holland) in cyclic voltammetry/linear sweep voltammetry mode. The electrodes were immersed in the relevant quiescent solution whilst the potential was scanned at a particular sweep rate and the current recorded by the software. For cyclic voltammetry with tetrathiafulvalene, all solutions were kept at 25°C constant temperature, in the dark, with a blanket of nitrogen.

2.2.17. Amperometric Procedures.

A three electrode system was used in conjunction with an Autolab (GPES3 software, EcoChemie B.V., Utrecht, Holland) or a precision potentiostat (Ministat, H. B. Thompson and Associates, Newcastle-upon-Tyne). The current was recorded by the Autolab program (in amperometric mode) and then printed out on a dot-matrix printer (Epson UK Ltd., Hertfordshire). The Ministat output was linked to an x-y-t chart recorder (Kipp and Zonnen Ltd., Sough, Berkshire) via a resistor board (JJ Instruments, Southampton, Hampshire). A saturated calomel reference electrode (SCE from Russell, Auchtermuchty, Fife) and an auxiliary Pt wire electrode (0.5 mm diameter, BDH, Poole, Dorset) were used with the working electrode in a beaker containing 5 or 10 ml of stirred solution. Measurements were taken at ambient temperature and pressure unless otherwise stated.

2.2.18. Calibrations.

Hydrogen peroxide, L-lactate and ascorbic acid calibrations were carried out as follows. The working, reference and counter electrodes were immersed in 10 ml of stirred buffer (containing electrolyte, either 0.1 M KCl or 0.15 M NaCl) and operating potential applied until a steady baseline was observed. This being reached, a known volume of sample solution (either buffer containing 10 M hydrogen peroxide (BDH Limited, Poole, Dorset), 0.2 M lithium L-lactate (2-hydroxypropionic acid from Sigma Chemical Company, London), or 0.5 M sodium salt of L-ascorbic acid (Sigma Chemical Company, London)) was injected into the buffer at a distance from the working electrode. When a steady current was again reached, another aliquot of sample solution was added, resulting in a step-like response.

2.2.19. Determination of Electrochemical Surface Areas.

Potential step chronocoulometry is one method for electrochemical determination of electrode area and is calculated from the integrated Cottrell equation (Bard & Faulkner, 1980):

$$Q=\frac{2.n.F.A.D.^{1/2}C.t^{1/2}}{\pi^{1/2}}$$

Where

Q	total charge at time t (in seconds)
n	number of electrons
F	Faraday constant (96485 $C.mol^{-1}$ electrons)
A	electrochemical area (cm^2)
D	diffusion coefficient for the redox species ($cm^2.s^{-1}$)
C	concentration of redox species ($mol.cm^{-3}$)
t	total time of potential step (in seconds)

Additional components arising from double layer charging and adsorbed species also contribute to the total charge. A plot of charge as a function of $t^{1/2}$ gives a linear gradient with intercept arising from the additional components. The electrochemical surface area of the electrodes was calculated from the gradient of the charge versus $t^{1/2}$ line using a diffusion coefficient for hexacyanoferrate (II) of 0.632×10^{-5} $cm^2.s^{-1}$ (Von Stackelberg *et al.*, 1953).

Prior to chronocoulometry the hand-fabricated and platinum electrodes were polished with silicon carbide paper (1200 grade, RS Components, Corby, Northamptonshire), 15 µm diamond paste (BAS, Bioanalytical Systems, Inc., Indiana, USA) and 0.3 µm alumina (BDH, Poole, Dorset) with sonication for 1 minute in water between each polishing material. The electrode was then cleansed with nitric acid (30% by volume diluted from 60% by volume, Analytical grade from BDH, Poole, Dorset) and sonicated again for 1 minute in water. The screen-printed electrodes were placed in an oven at 60°C for approximately 10 hours in order to remove moisture and cake the carbon onto the base electrode. A three electrode system comprising a saturated Calomel reference electrode, a platinum wire auxiliary electrode and the working electrode was immersed in 5 ml of quiescent

potassium hexacyanoferrate (II) solution (0.01 M in 1 M KCl solution) in the dark at 25°C. The potential was poised at 0 V (SCE) and then increased to +450 mV (SCE) for 500 ms whilst the charge was recorded. The solution and working electrode was then changed and the procedure repeated, except for the platinum disc electrode which was cleaned with nitric acid and sonicated between each of five measurements.

The potassium hexacyanoferrate (II) solution was diluted to 0.05 M in 1 M KCl and the above procedure repeated. The electrochemical surface area of five carbon-foil electrodes and the platinum disc electrode (with cleaning in between each measurement) was determined.

The recorded charge data were imported into the report mode of the GPES3 program in order to calculate the slope of the charge versus square root of time plot. The straight portion of the line was taken for each set of data. These values were then averaged before placing in the integrated Cottrell equation to calculate the electrochemical surface area for each type of electrode.

2.2.20. Measurement and Presentation of Results.

Amperometric traces were obtained and the current increase form the background was measured (generally after 30 s). All data were then recorded on Excel spreadsheets (Microsoft version 5.0 for Windows) and the average, standard deviation and standard error were calculated from accumulated data sets. The average (mean), standard deviation and standard error are given as:

$$\bar{x} = \frac{\sum x}{n} \qquad \vartheta_{n-1} = \sqrt{\frac{\Sigma(x - mean)^2}{n-1}} \qquad S.E. = \frac{\vartheta_{n-1}}{\sqrt{n}}$$

respectively. The error bars in all graphs respresent the standard error of the mean response unless otherwise stated. Error bars at zero concentration are the standard error of the background reading (which was used as the zero baseline). The coefficient of variation was calculated as:-

$$\frac{\vartheta_{n-1}}{\bar{x}} \times 100\%$$

2.3. RESULTS.

2.3.1. Hexacyanoferrate (III) Modification.

Typical cyclic voltammograms during modification of carbon electrodes with hexacynaoferrate (III) are shown in Figure 2.5. It can be seen that there is only one peak on the second scan at approximately +375 mV (SCE) but upon modification, two new peaks appear at approximately +0.7 and +1.15 V (SCE). It can also be seen that the current at +2.5 V (SCE) has decreased.

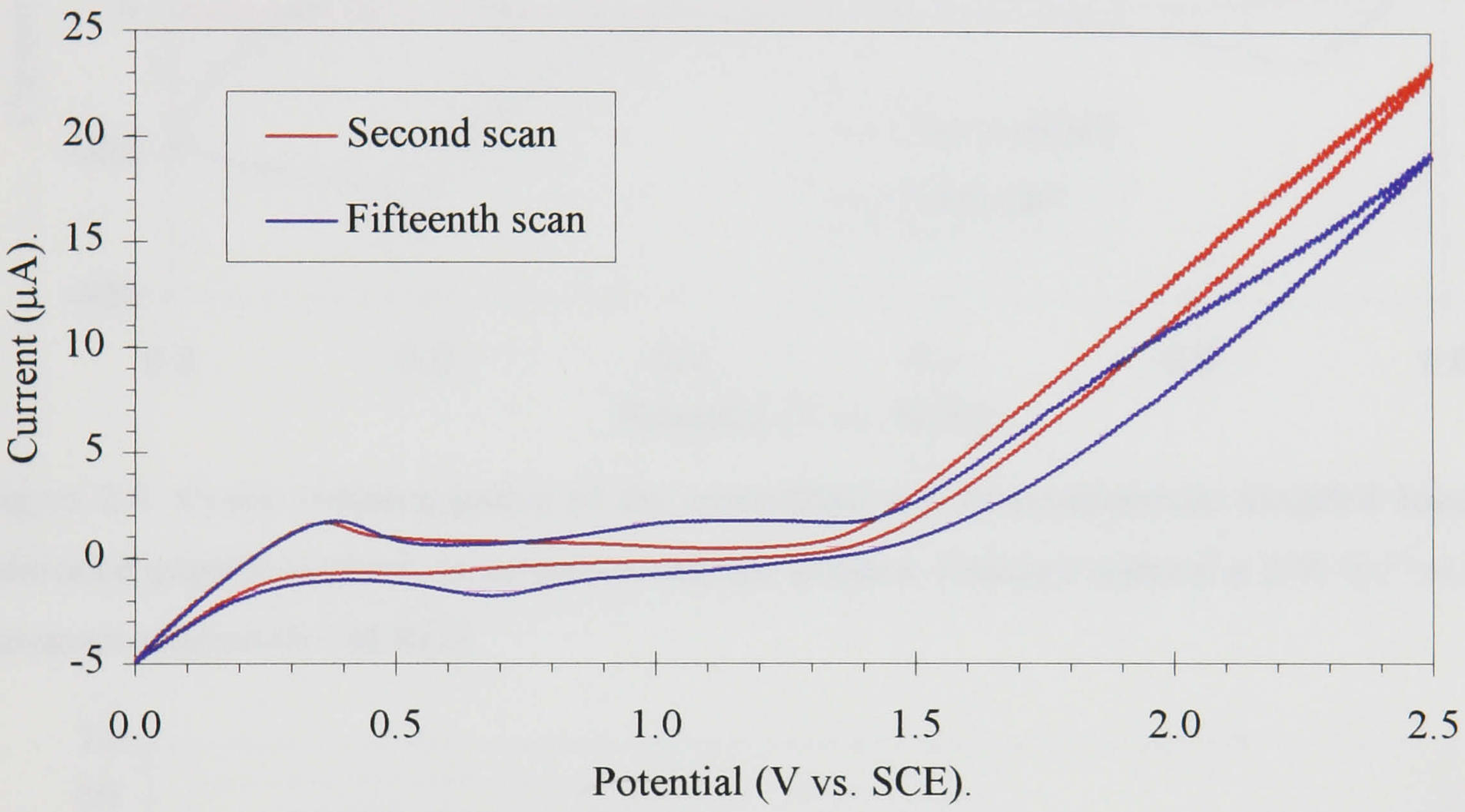


Figure 2.5. Cyclic voltammograms of a hand fabricated carbon graphite electrode modification with hexacyanoferrate film. Potential scanned at 0.05 $V.s^{-1}$ in a stirred potassium hexacyanoferrate (III) solution (0.1 M).

The cyclic voltammograms shown in Figure 2.6. were carried out in 0.1 M KCl using one hand fabricated electrode, before and after modification with hexacyanoferrate (III). The modified electrode shows two sets of peaks at approximately +0.2 V and +0.9 V (SCE) and the oxidation and reduction peaks are separated by 20 mV and 25 mV respectively. Upon the addition of lactate oxidase to the solution, no changes in peak heights or positions were observed.

Hydrodynamic voltammograms of six hand fabricated electrodes modified with hexacyanoferrate (III) tested with hydrogen peroxide are shown in Figure 2.7. It can be seen that electro-oxidation of hydrogen peroxide occurs above +300 mV (SCE) and reduction occurs below this potential.

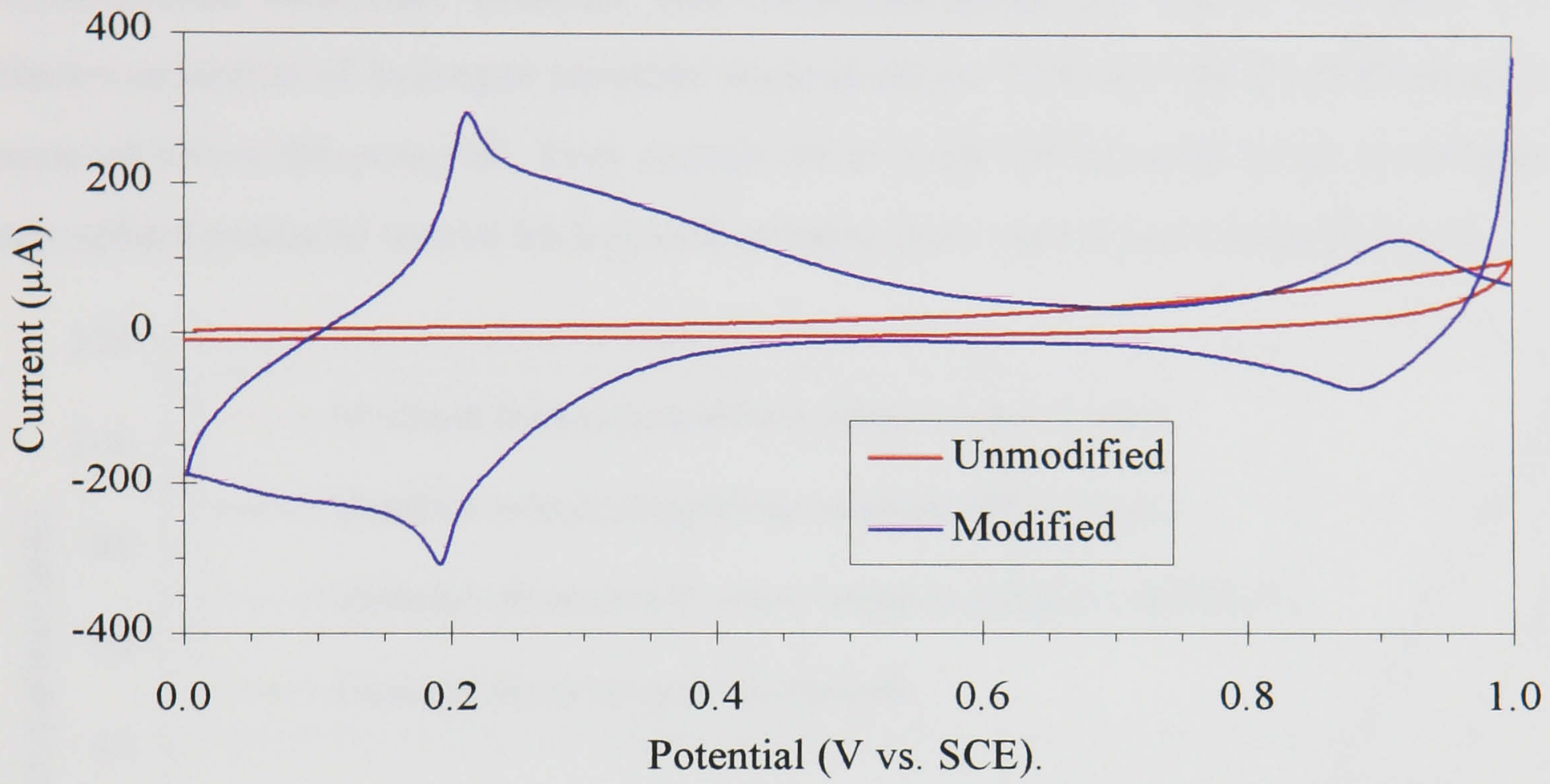


Figure 2.6. Cyclic voltammograms of the unmodified and hexacyanoferrate-modified hand-fabricated graphite electrode in potassium chloride solution. Potential scanned at 0.05 $V.s^{-1}$ in a quiescent solution (0.1 M KCl).

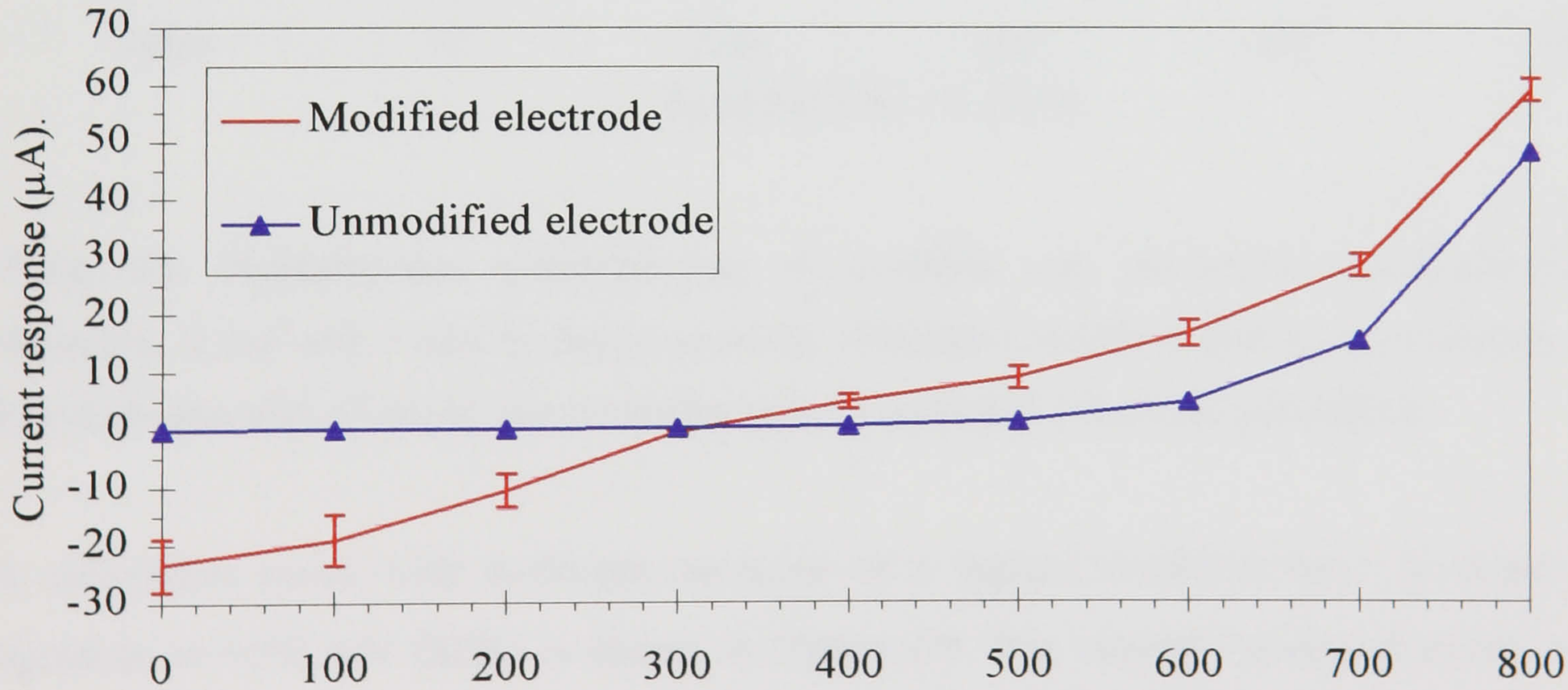


Figure 2.7. Hydrodynamic voltammogram of hexacyanoferrate (III)-modified electrodes tested with 5 mM hydrogen peroxide. Six carbon hand fabricated electrodes, 15 scans modification in potassium hexacyanoferrate (III) (0.1 M).

Screen-printed electrodes with a working surface of graphite ink made using cellulose acetate were successfully modified with hexacyanoferrate (III) using the cyclic voltammetric procedure. Other electrodes made with commercial carbn inks or carbon in hydroxyethyl cellulose, were unsuccessful. The hydrodynamic voltammagrams of screen-printed electrodes modified with hexacyanoferrate are shown in Figure 2.8. Electro-oxidation of hydrogen peroxide occured above +200 mV (SCE) and reduction occurred below this potential. Tests carried out at 0 mV (SCE) under an air or nitrogen atmosphere produced similar background currents (zero current) and noise (0.3 μA).

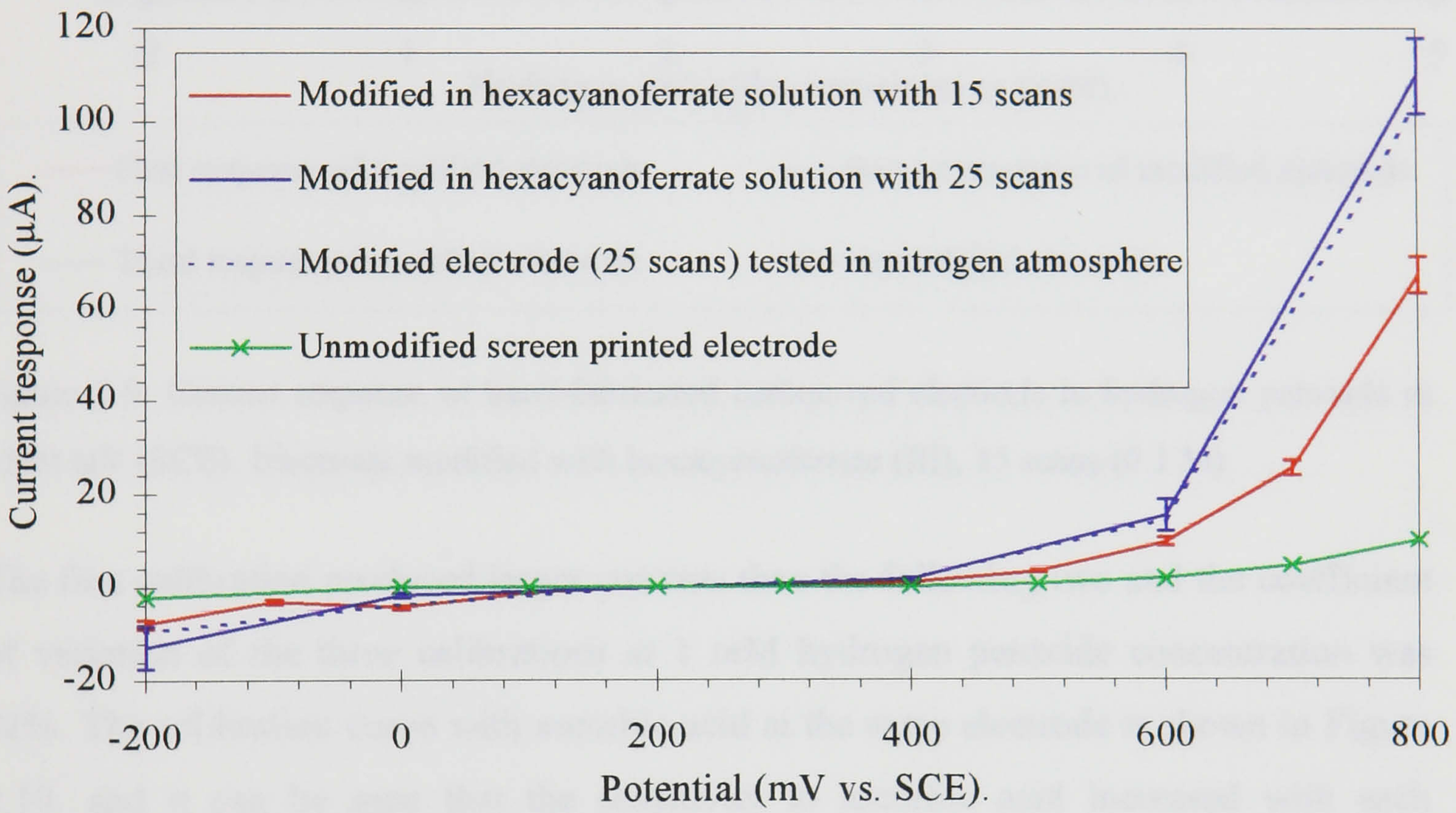


Figure 2.8. Hydrodynamic voltammogram of modified and unmodified screen-printed electrodes, tested with 5 mM hydrogen peroxide. Electrodes modified with hexacyanoferrate, four electrodes with 15 scans, two electrodes with 25 scans and 1 electrode unmodified.

A calibration curve with hydrogen peroxide of a typical modified hand fabricated electrode at +500 mV (SCE) is shown in Figure 2.9. The current density observed at +500 mV to hydrogen peroxide was 0.01 $\mu A.mM^{-1}.mm^{-2}$ for hexacyanoferrate (III)-modified electrodes and 0.00 $\mu A.mM^{-1}.mm^{-2}$ for unmodified electrodes.

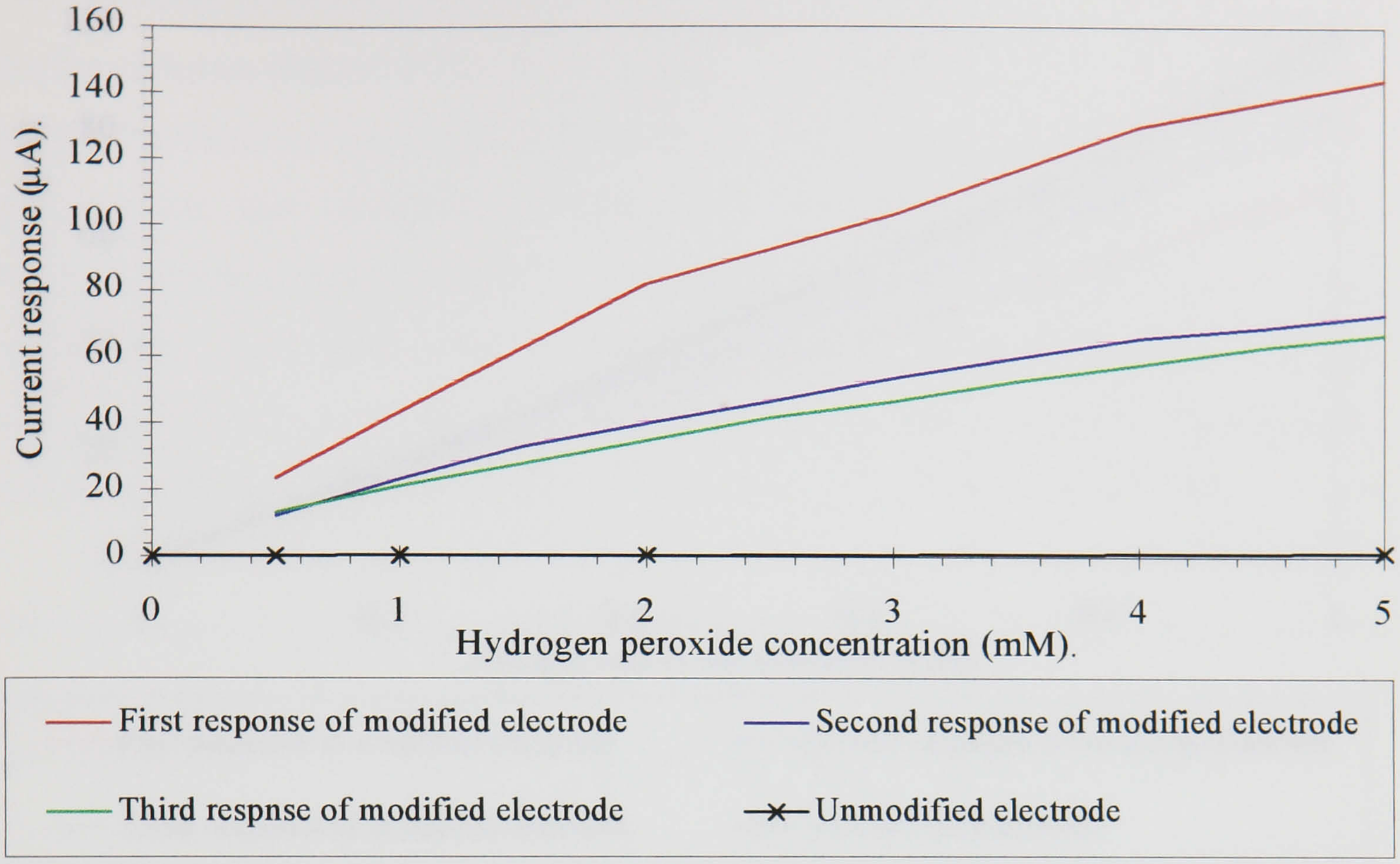


Figure 2.9. Current response of hand-fabricated carbon-rod electrode to hydrogen peroxide at +500 mV (SCE). Electrode modified with hexacyanoferrate (III), 15 scans (0.1 M).

The first calibration produced larger currents than the following two and the coefficient of variation of the three calibrations at 1 mM hydrogen peroxide concentration was 42%. The calibration curve with ascorbic acid at the same electrode is shown in Figure 2.10. and it can be seen that the sensitivity to ascorbic acid increased with each calibration. The modified electrode was more sensitive to hydrogen peroxide and less sensitive to ascorbic acid than an unmodified electrode.

Figure 2.11. shows calibration curves for two typical hexacyanoferrate (III)-modified screen-printed electrodes with hydrogen peroxide at +500 mV (SCE). The electrodes gave a very wide dynamic range. The current density observed at +500 mV to hydrogen peroxide was 0.01 $\mu A.mM^{-1}.mm^{-1}$ for a hexacyanoferrate (III)-modified electrode and 0.00 $\mu A.mM^{-1}.mm^{-1}$ for an unmodified electrode. It was noted that the hexacyanoferrate (III)-modified electrodes gave varying responses, both between electrodes and within electrodes. The coefficient of variation at 5 mM hydrogen peroxide for 6 electrodes modified with 15 scans in hexacyanoferrate was 67% and for two electrodes modified with 25 scan in hexacyanoferrate was 49%.

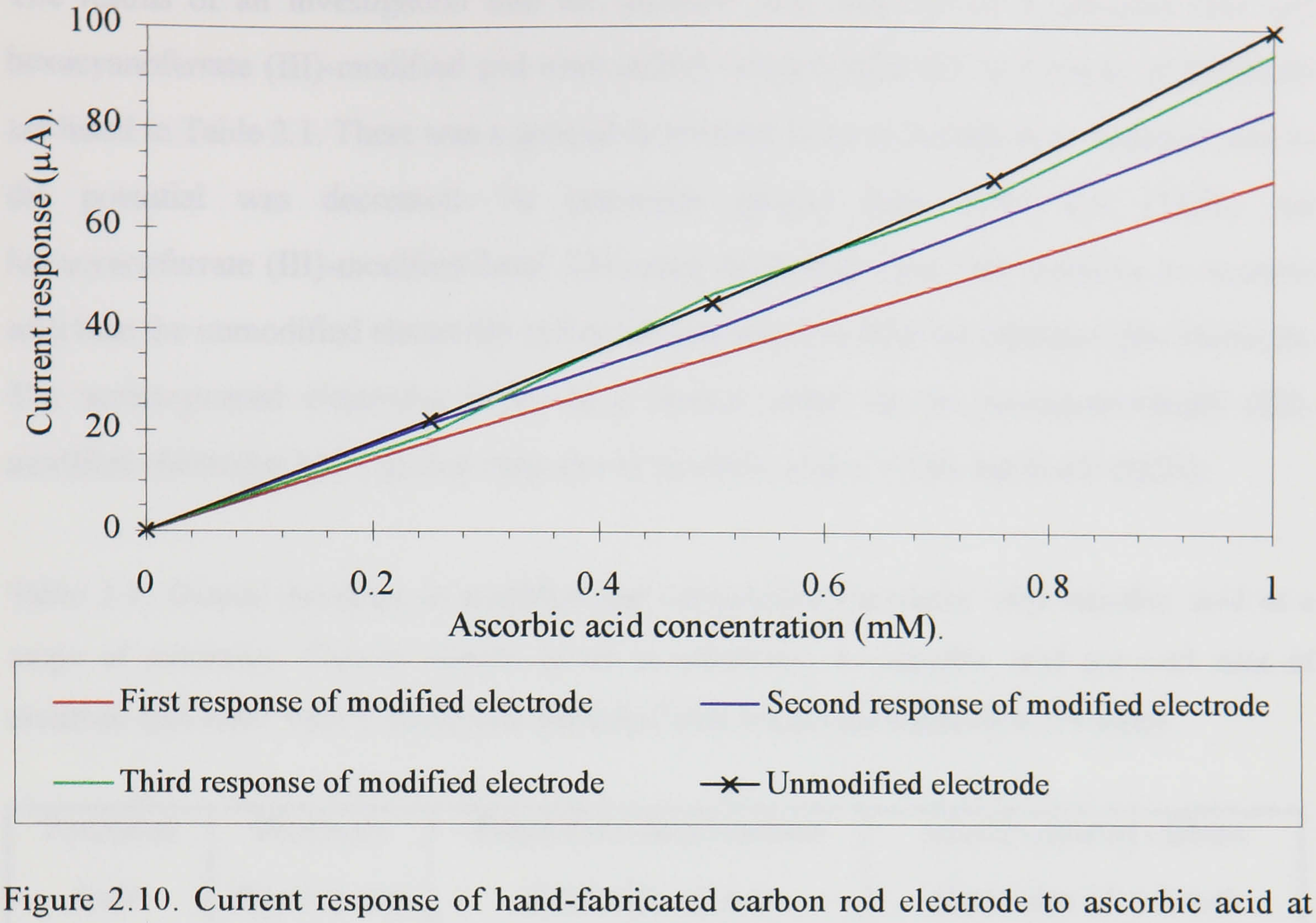


Figure 2.10. Current response of hand-fabricated carbon rod electrode to ascorbic acid at +500 mV (SCE). Electrode modified with hexacyanoferrate (III), 15 scans (0.1 M).

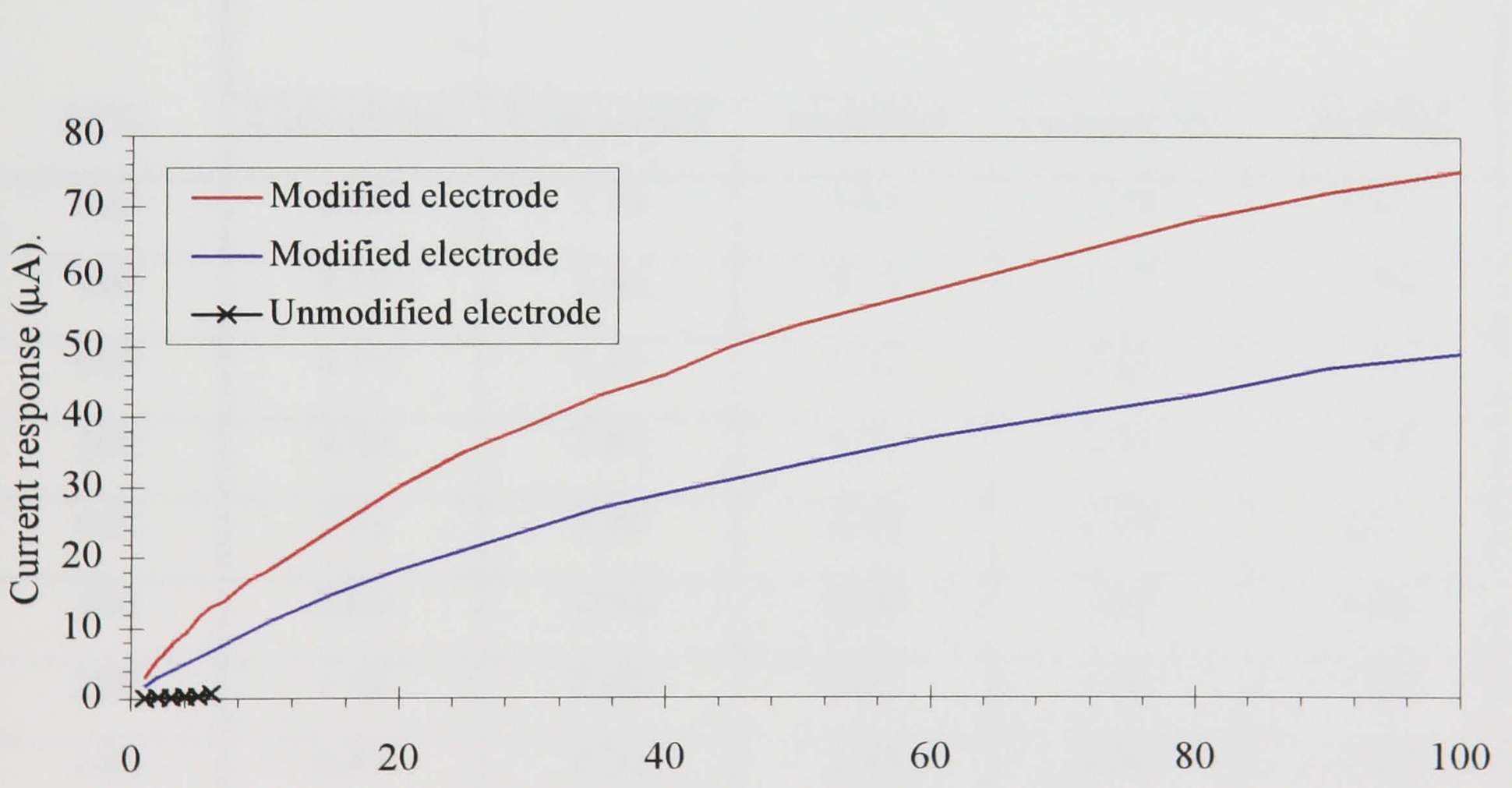


Figure 2.11. Individual responses of modified and unmodified screen-printed electrodes to hydrogen peroxide at +500 mV (SCE). Electrodes modified with hexacyanoferrate film, 15 scans on each electrode.

The results of an investigation into the ascorbic acid response of a platinum disc and hexacyanoferrate (III)-modified and unmodified carbon electrodes at a range of potentials are listed in Table 2.1. There was a general downward trend in sensitivity to ascorbic acid as the potential was decreased. At potentials greater than +400 mV (SCE), the hexacyanoferrate (III)-modified hand fabricated electrodes were less sensitive to ascorbic acid than the unmodified electrodes and much less sensitive than the platinum disc electrode. The screen-printed electrodes followed a similar trend but the hexacyanoferrate (III)-modified electrodes had a greater response to ascorbic acid at +100 and 0 mV (SCE).

Table 2.1. Current densities of modified and unmodified electrodes with ascorbic acid at a range of potentials. Current density given as sensitivity to ascorbic acid per unit area of electrode ($\mu A.mM^{-1}.mm^{-1}$). Electrodes modified with hexacyanoferrate (III), 15 scans.

Potential (mV versus SCE)	Platinum disc 1.6 mm diameter	Hand-fabricated carbon electrodes (6 mm diameter)		Screen-printed carbon electrodes (7 x 2 mm surface area)	
	Unmodified	Unmodified	Modified	Unmodified	Modified
800	4.63	3.18	3.01	3.25	1.97
700	5.30	3.46	3.11	3.17	1.76
600	4.57	3.61	3.11	2.68	1.65
500	4.56	3.35	3.11	2.51	1.40
400	3.51	3.36	2.90	2.19	1.43
300	2.60	2.94	3.29	1.84	1.38
200	1.45	2.60	3.22	1.45	1.22
100	0.47	1.56	2.69	0.89	0.95
0	0.00	0.76	1.43	0.34	0.79

Lactate oxidase was immobilised onto hexacyanoferrate (III)-modified hand fabricated electrodes using carbodiimide. The response to L-lactate at +500 mV (SCE) of three electrodes is shown in Figure 2.12. It can be seen that there was some variation between the sensors and the individual response also varied. The coefficient of variation at 5 mM L-lactate concentration for electrode 1, 2 and 3 were 4%, 18% and 30% respectively. The coefficient of variation between the three sensors at 5 mM L-lactate was 52%. The sensors had a large dynamic range but a very short linear range. Unfortunately, none of the sensors were calibrated from 0 to 1 mM L-lactate but the average sensitivity from 0-5 mM was calculated as 0.79 $\mu A.mM^{-1}$ L-lactate. The sensors gave a 90% response to 5 mM L-lactate in less than 20 seconds.

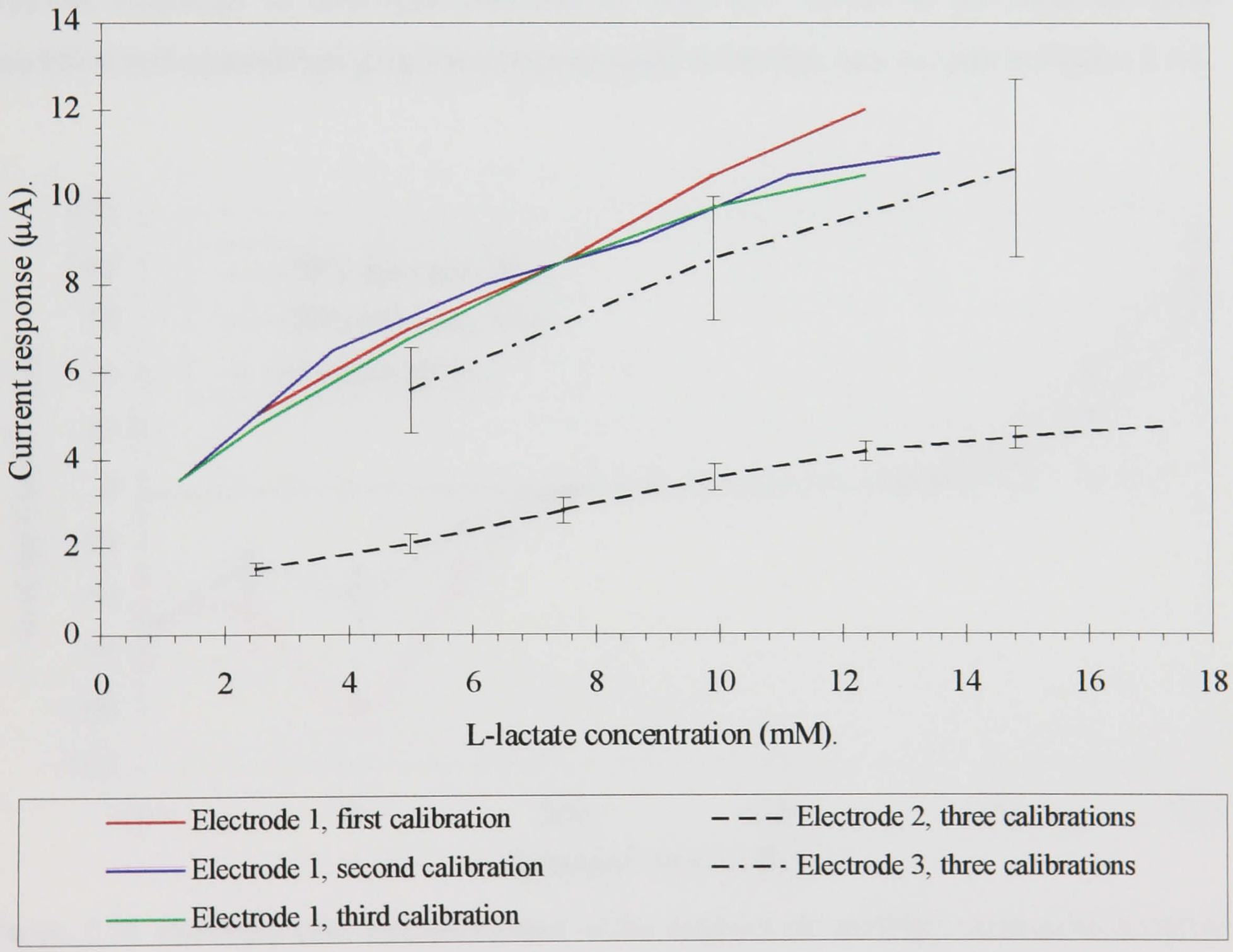


Figure 2.12. Response to L-lactic acid at +500 mV (SCE) of three modified hand-fabricated carbon electrodes. Electrodes modified with hexacyanoferrate (III), 15 scans, and lactate oxidase.

2.3.2. Prussian Blue-Modified Electrodes.

A cyclic voltammogram of Prussian Blue in potassium chloride solution (0.1 M) with a screen-printed carbon electrode gave peaks at approximately 0.3 and 0.1 V (SCE), consistent with the ferro/ferri cyanide redox couple. Scanning the electrode afterwards revealed the same peaks but much smaller and decreasing with time.

Screen-printed electrodes modified with a Prussian Blue graphite ink applied to the working surface gave hydrodynamic voltammograms as illustrated in Figure 2.13. It can be seen that electro-oxidation began to take place above +500 mV (SCE) and electro-reduction below +190 mV (SCE).

Typical responses to hydrogen peroxide at +600 mV (SCE) at the Prussian Blue-modified and unmodified graphite screen-printed electrodes can be seen in Figure 2.14.

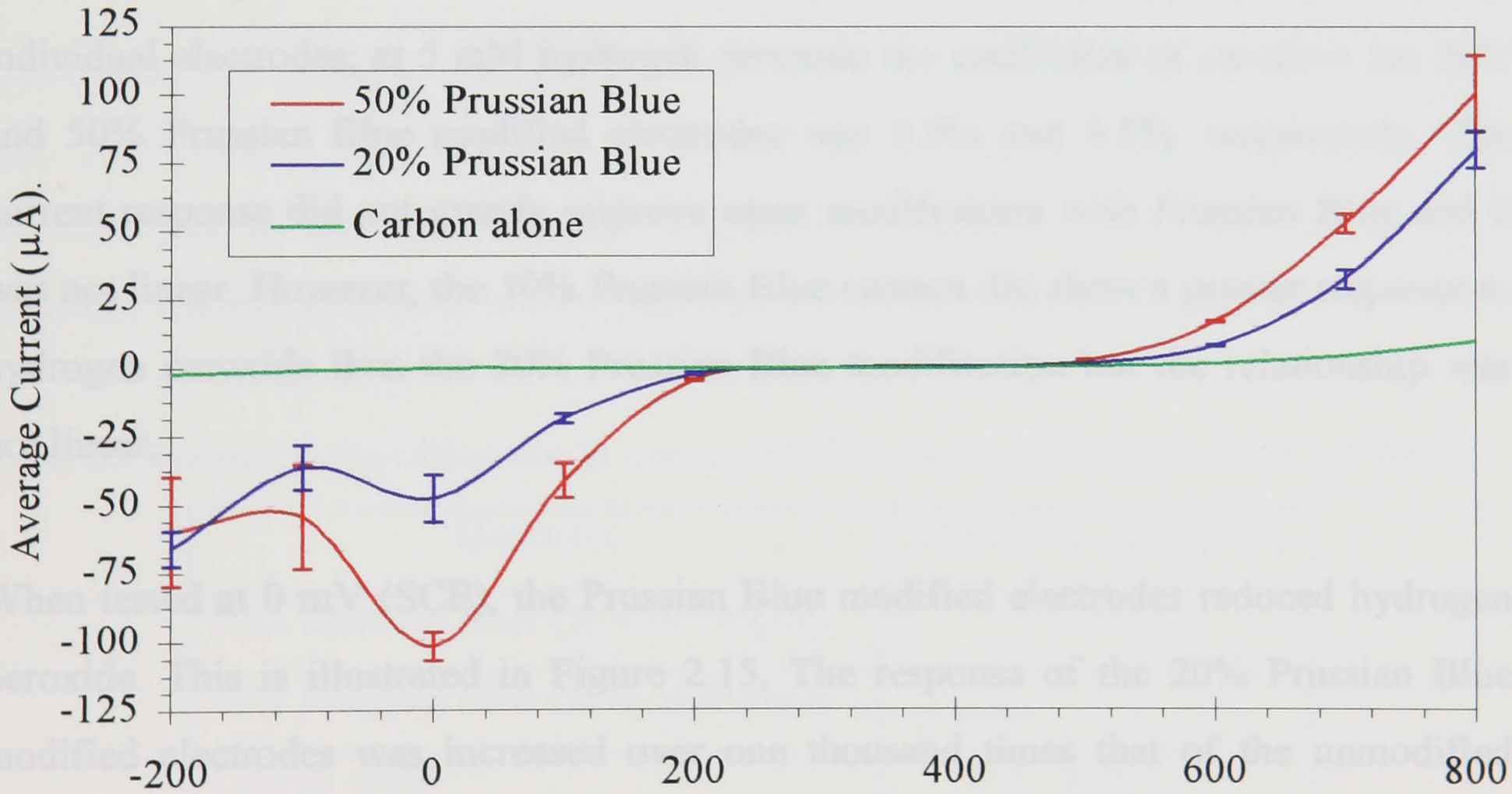


Figure 2.13. Hydrodynamic voltammograms of the response of modified screen-printed carbon electrode to 5 mM hydrogen peroxide. Carbon ink modified with Prussian Blue (20 or 50 % w/w), three electrodes of each modification tested.

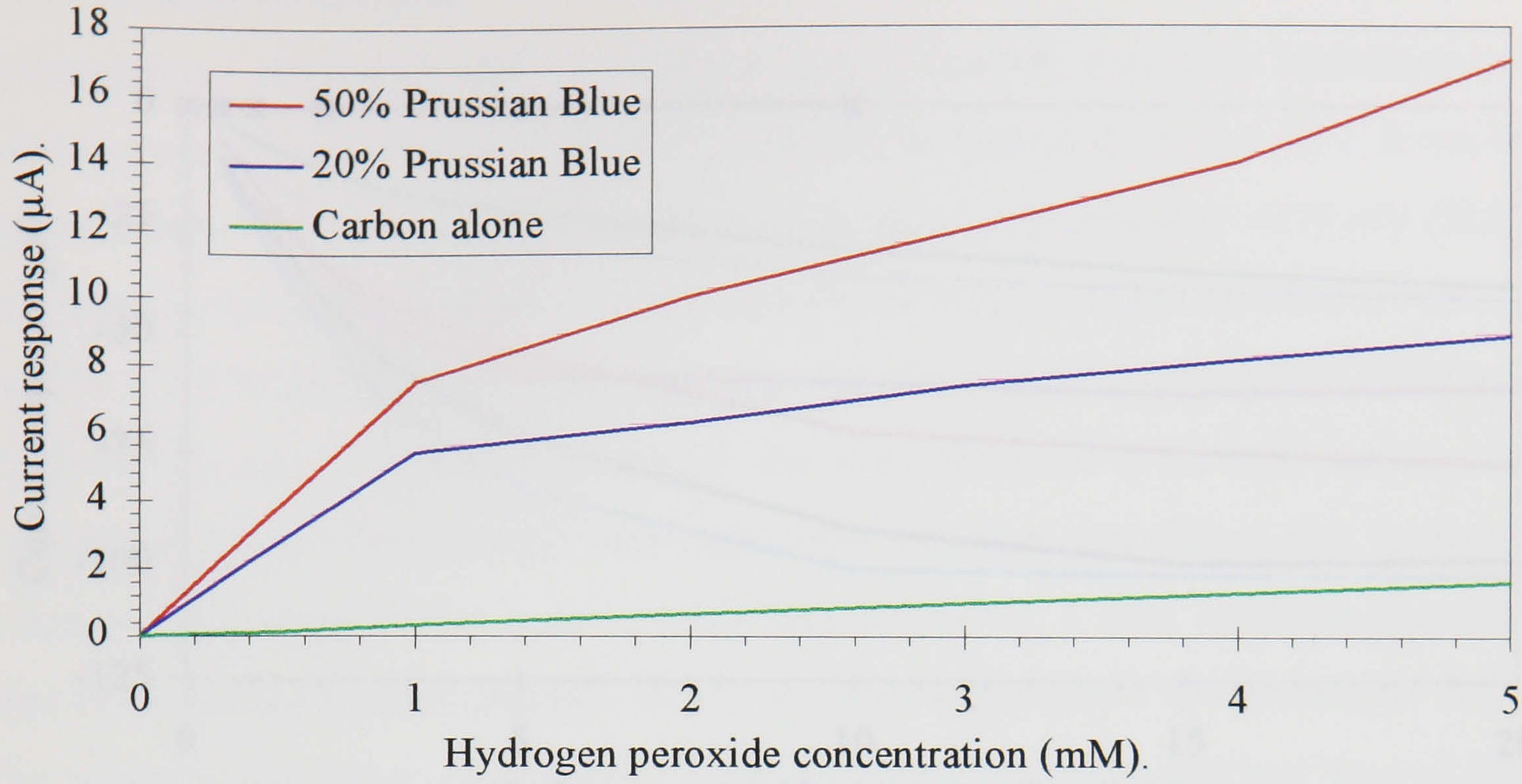


Figure 2.14. Response of modified screen-printed electrodes to hydrogen peroxide at +600 mV (SCE). Prussian Blue modified carbon ink.

There was only a small variation between first calibrations with hydrogen peroxide on individual electrodes; at 5 mM hydrogen peroxide the coefficient of variation for 20% and 50% Prussian Blue modified electrodes was 9.0% and 4.5%, respectively. The current response did not greatly improve upon modification with Prussian Blue and it was not linear. However, the 50% Prussian Blue content did show a greater response to hydrogen peroxide than the 20% Prussian Blue modification but the relationship was not linear.

When tested at 0 mV (SCE), the Prussian Blue modified electrodes reduced hydrogen peroxide. This is illustrated in Figure 2.15. The response of the 20% Prussian Blue modified electrodes was increased over one thousand times that of the unmodified electrode and the response from 50% Prussian Blue modified electrodes was increased nearly three thousand times. However, there was great variation in the response given by different electrodes and indeed by an individual electrode, for both 20% and 50% Prussian Blue content. The coefficients of variation with 5 mM hydrogen peroxide for six 20% and five 50% Prussian Blue-modified electrodes were 51% and 65%, respectively.

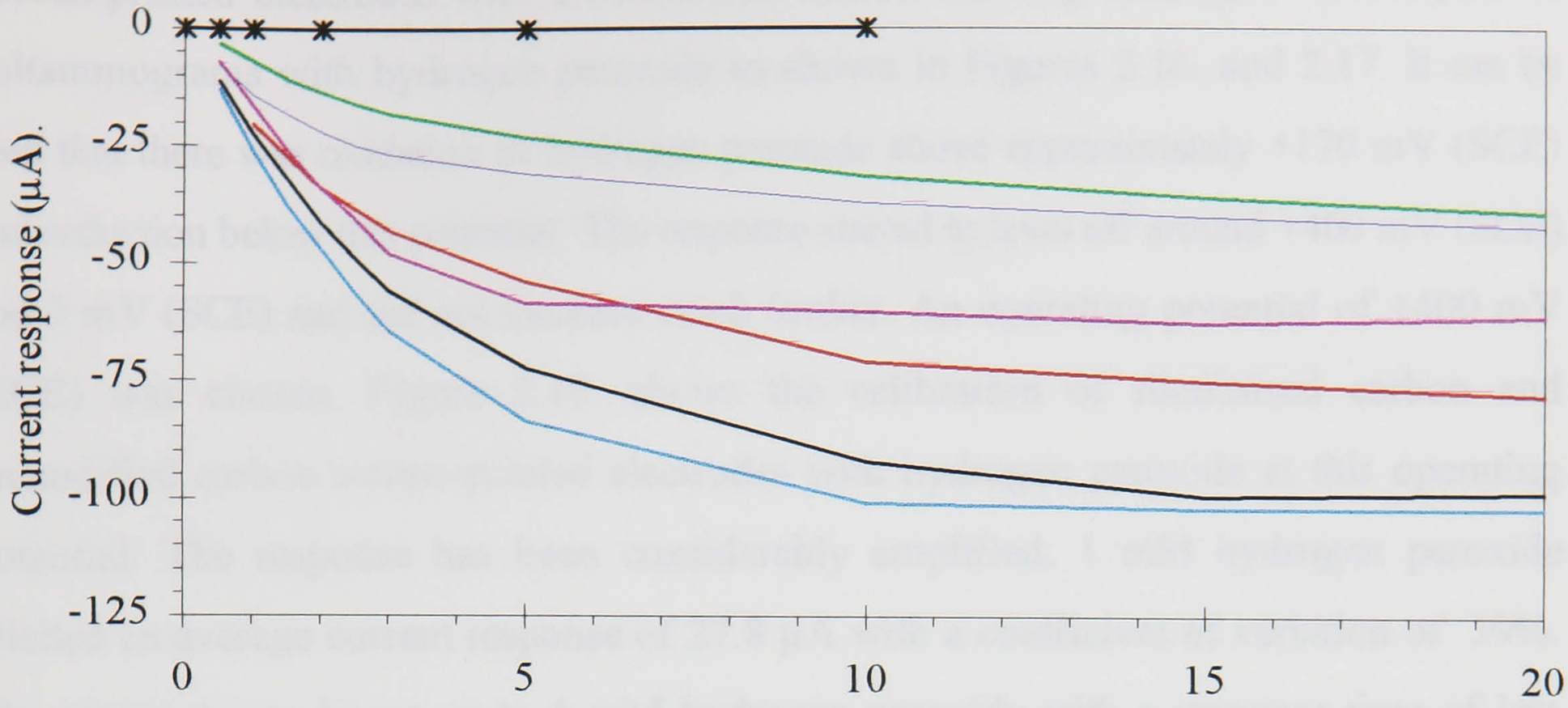


Figure 2.15. Response of modified carbon electrodes to hydrogen peroxide at 0 mV (SCE). Carbon ink modified with 20% Prussian Blue (w/w of carbon), the response of six different electrodes are shown. The response from an unmodified electrode is represented by *.

The response to ascorbic acid was also measured. The results are summarized in Table 2.2. The Prussian Blue-modified screen-printed electrodes were more sensitive to ascorbic acid at this potential than unmodified electrodes, although the modified electrode containing more Prussian Blue was not proportionally more sensitive.

Table 2.2. Sensitivity of modified-carbon screen-printed electrodes to ascorbic acid at 0 mV (SCE).

Electrode	Sensitivity ($\mu A.mM^{-1}$)
50% Prussian Blue (w/w of carbon)	9.46
20% Prussian Blue (w/w of carbon)	7
Carbon alone	4.76

2.3.3. Rhodinised Carbon.

Screen-printed electrodes with a rhodinised carbon working area gave hydrodynamic voltammograms with hydrogen peroxide as shown in Figures 2.16. and 2.17. It can be seen that there was oxidation of hydrogen peroxide above approximately +170 mV (SCE) and reduction below this potential. The response started to level off around +400 mV (SCE) and 0 mV (SCE) and did not increase much further. An operating potential of +400 mV (SCE) was chosen. Figure 2.18. shows the calibration of rhodinised carbon and unmodified carbon screen-printed electrodes with hydrogen peroxide at this operating potential. The response has been considerably amplified, 1 mM hydrogen peroxide elicited an average current response of 27.8 μA with a coefficient of variation of 39%. The response was linear up to 1 mM hydrogen peroxide with a response time of less than 20 seconds to gain 90% full response.

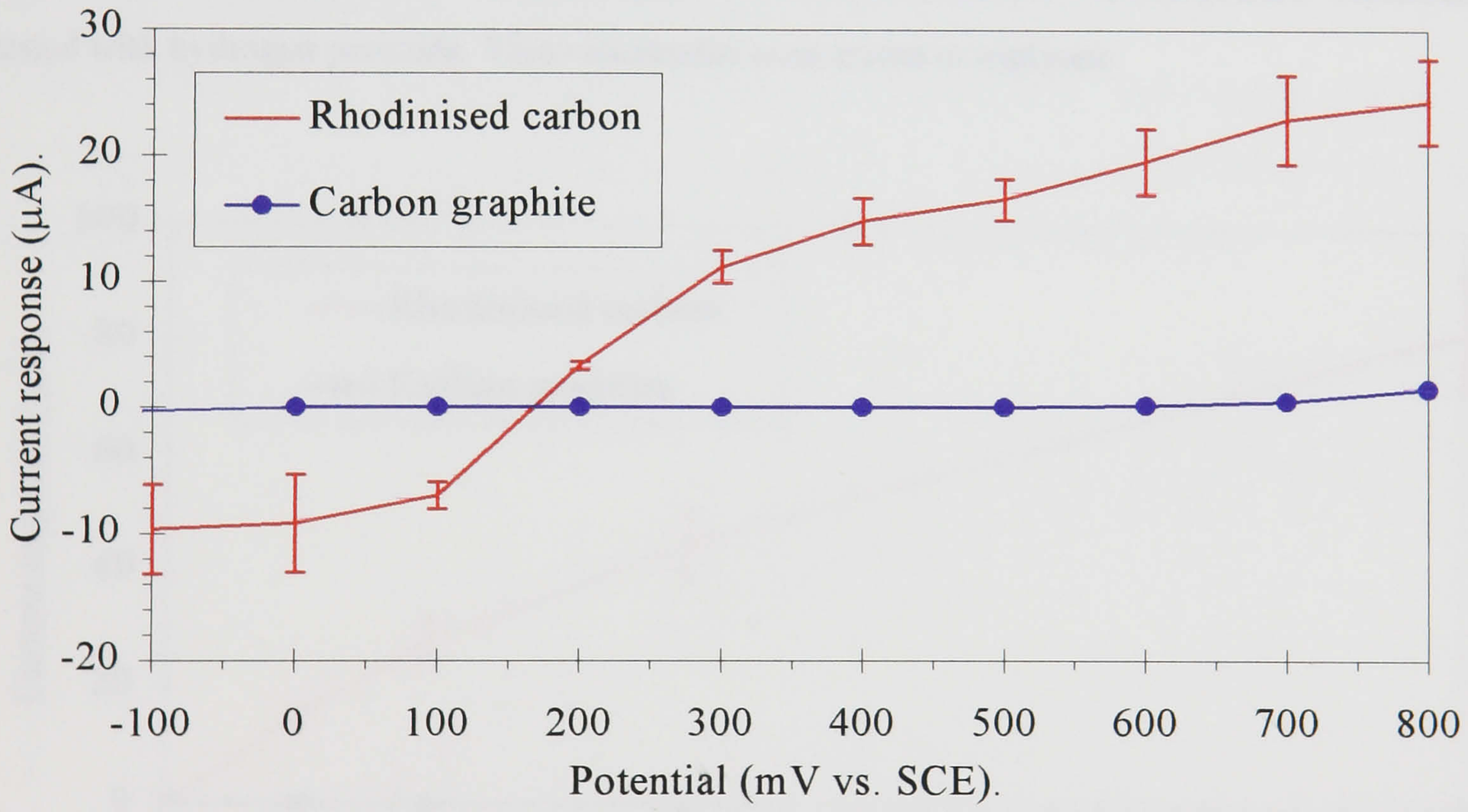


Figure 2.16. Hydrodynamic voltammogram of rhodinised-carbon or carbon-graphite screen-printed electrodes tested with 0.5 mM hydrogen peroxide. Eight rhodinised carbon electrodes and 1 carbon graphite electrode were tested.

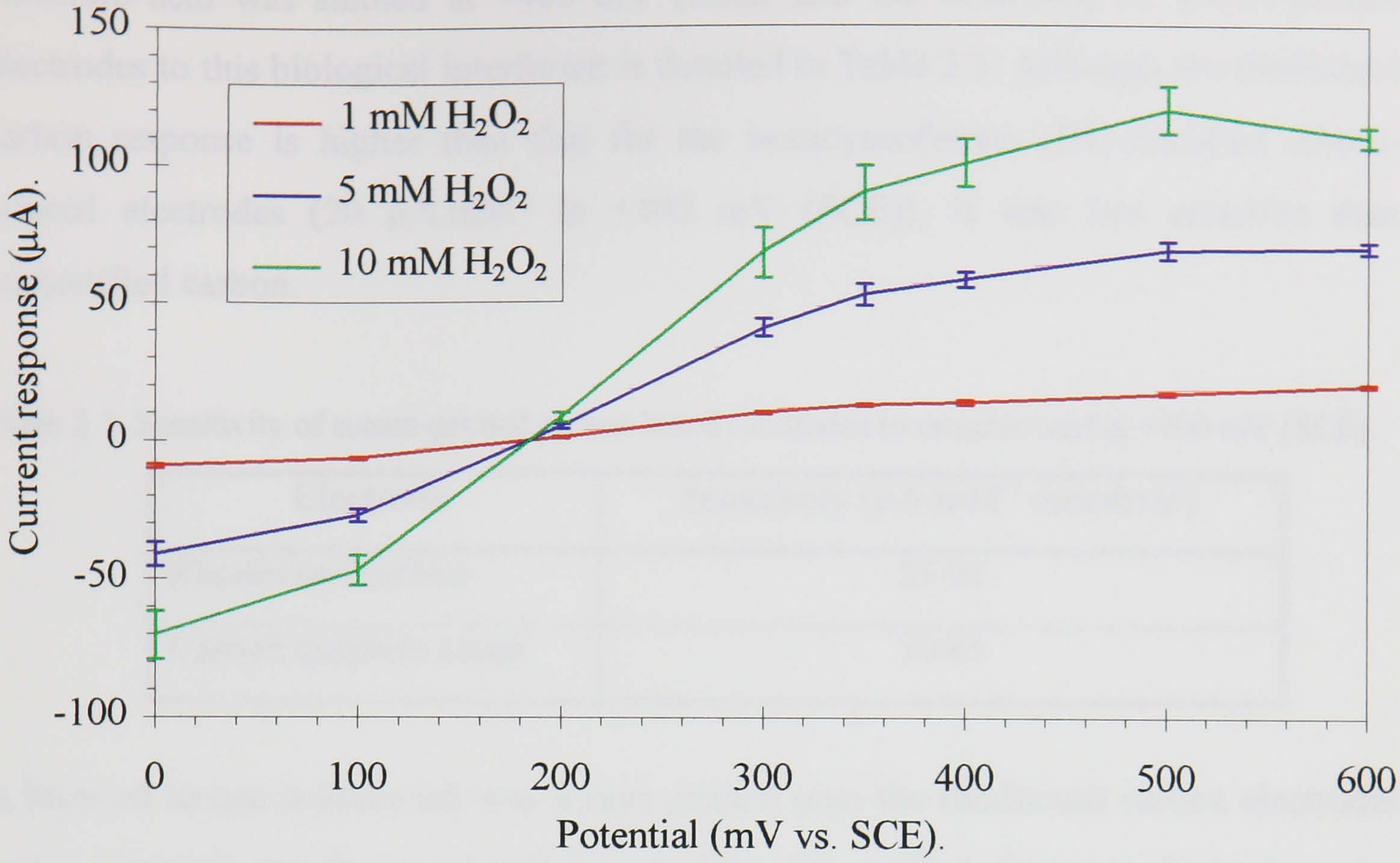


Figure 2.17. Hydrodynamic voltammogram of rhodinised-carbon screen-printed electrodes tested with hydrogen peroxide. Three electrodes were tested in triplicate.

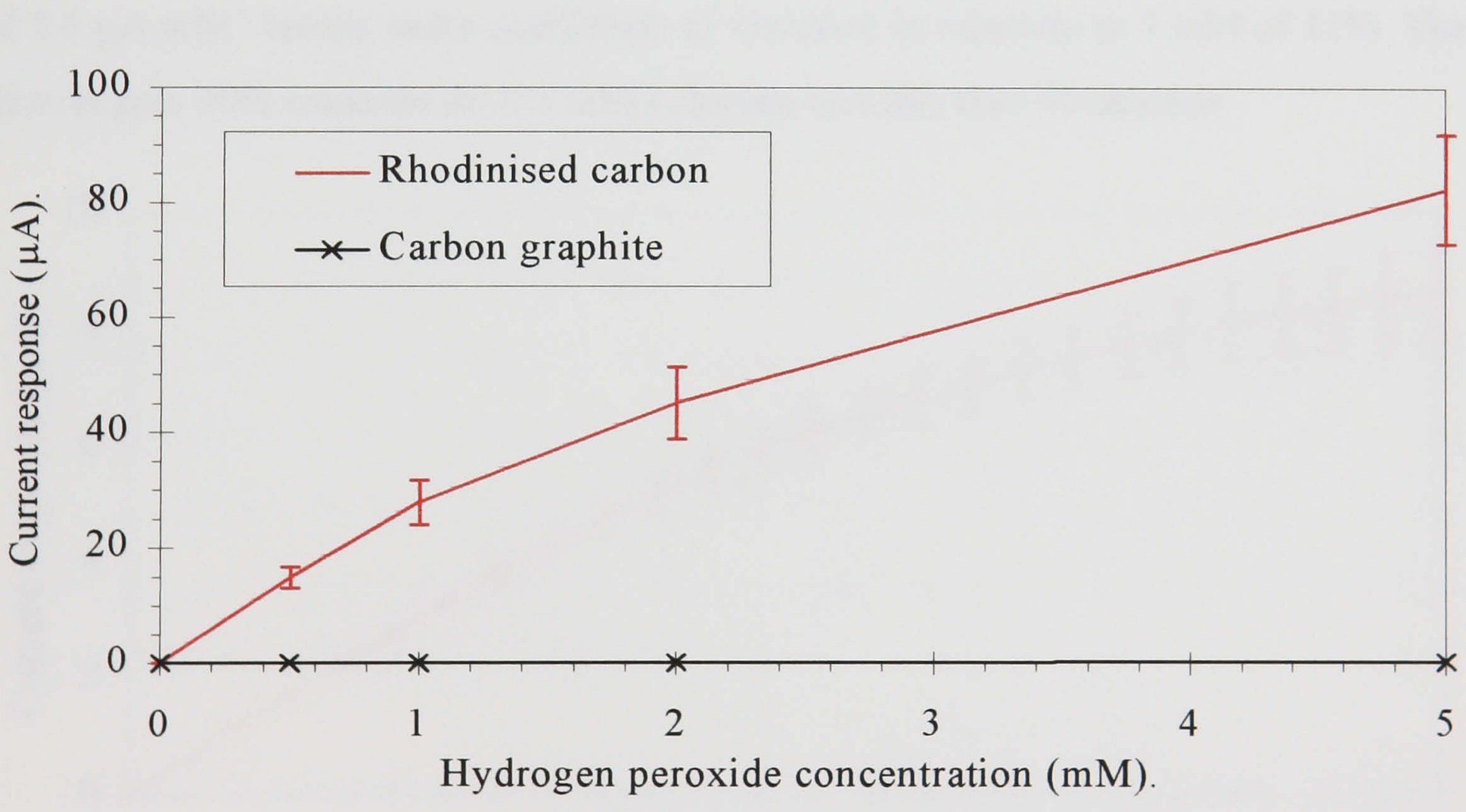


Figure 2.18. Detection of hydrogen peroxide at rhodinised-carbon or carbon-graphite screen-printed electrodes. Eight rhodinised carbon electrodes were tested at +400 mV (SCE).

Ascorbic acid was studied at +400 mV (SCE) and the sensitivity of screen-printed electrodes to this biological interferent is detailed in Table 2.3. Although the rhodinised carbon response is higher than that for the hexacyanoferrate (III)-modified screen-printed electrodes (20 $\mu A.mM^{-1}$ at +400 mV (SCE)), it was less sensitive than unmodified carbon.

Table 2.3. Sensitivity of screen-printed carbon based electrodes to ascorbic acid at +400 mV (SCE).

Electrode.	Sensitivity ($\mu A.mM^{-1}$ ascorbate).
Rhodinised carbon	25.91
Carbon graphite alone	30.65

A layer of lactate oxidase ink was screen printed onto the rhodinised carbon electrodes and a retaining membrane of cellulose acetate was applied. Enzyme electrodes were then tested in buffer with L-lactate at +400 mV (SCE) and a typical calibration is shown in Figure 2.19. The linear range of this sensor was 0 to 0.7 mM with a sensitivity of 5.6 $\mu A.mM^{-1}$ lactate and a coefficient of variation in response to 1 mM of 11%. The time to gain 90% response for 0.1 mM L-lactate was less than 40 seconds.

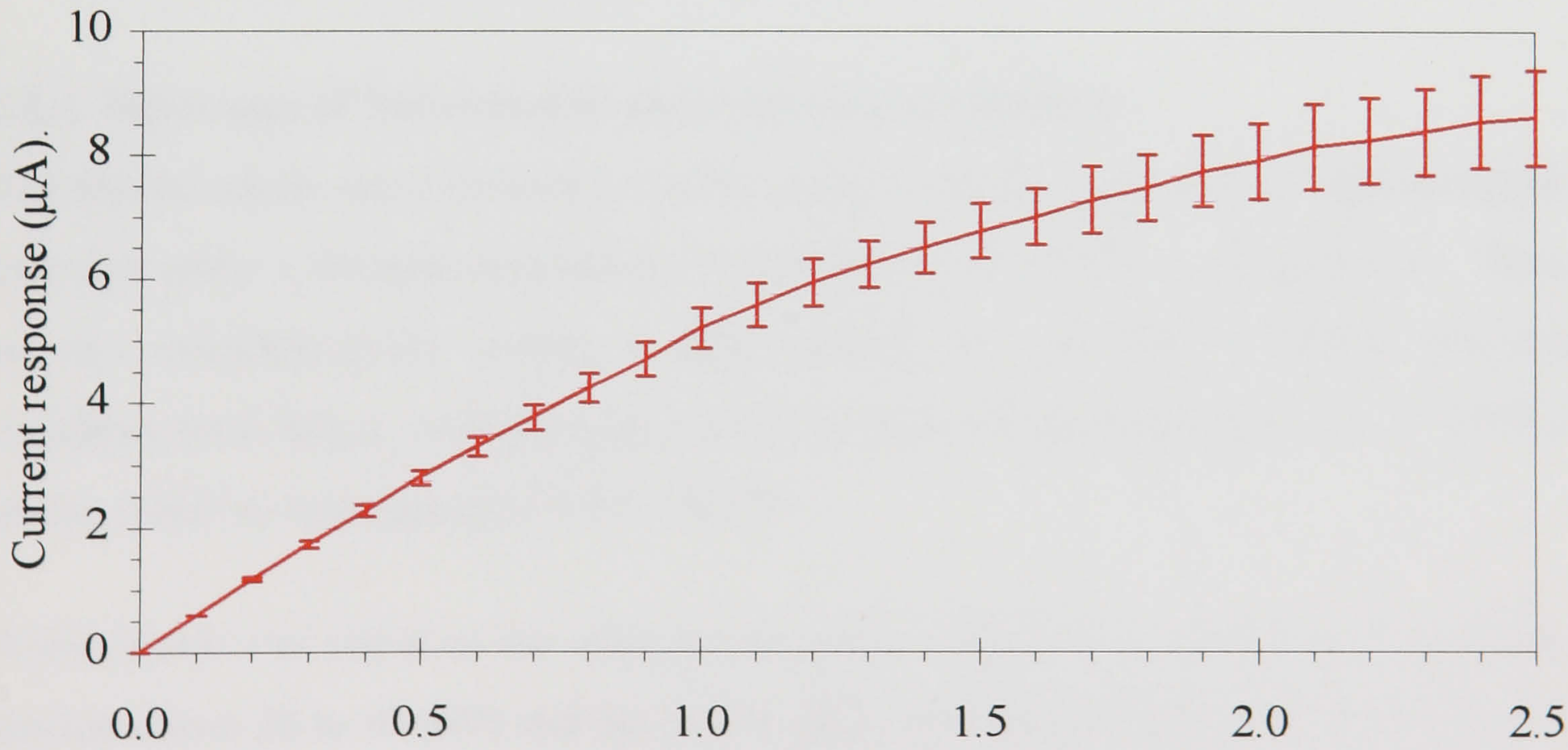


Figure 2.19. Response to L-lactate at +400 mV (SCE) of a modified-carbon screen-printed electrode. Three calibrations of an electrode modified with rhodinised carbon and lactate oxidase.

The sensors were very stable and varied little between electrodes. Figure 2.20. shows the calibration of three rhodinised carbon/lactate oxidase screen-printed electrodes at +400 mV (SCE). The linear range of these sensors is 0 to 0.7 mM with a sensitivity of 8.75, 7.0 and 6.0 $\mu A.mM^{-1}$ lactate for electrode 1, 2 and 3 respectively. The coefficient of variation between 1 mM L-lactate responses of these electrodes was 20%.

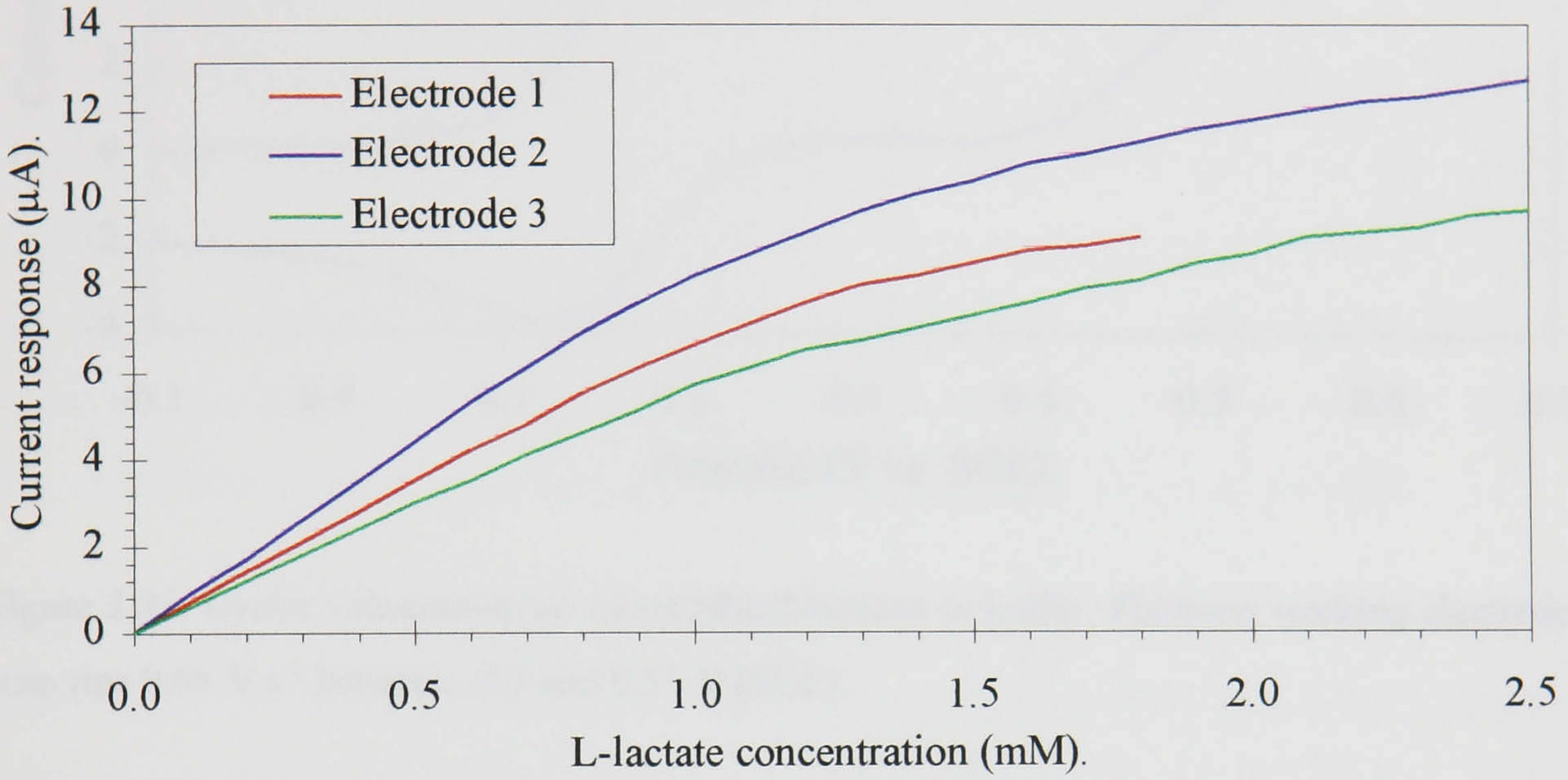


Figure 2.20. First calibration with L-lactate at +400 mV (SCE) of three screen-printed electrodes modified with rhodinised carbon and lactate oxidase.

2.3.4. Behaviour of Tetrathiafulvalene and Lactate Oxidase.

Tetrathiafulvalene was dissolved in buffer using Tween 80 and a cyclic voltammogram recorded under a nitrogen atmosphere. A typical result is shown in Figure 2.21. There are two oxidation peaks visible, at approximately 0.2 and 0.49 V (SCE). The first oxidation peak has a corresponding reduction peak at approximately 0.12 V (SCE), giving a half wave potential of 0.16 V (SCE).

When lactate was added to the solution, the peaks shifted to the left by an inconsistent amount (from 20 to 40 mV) and the peak heights increased slightly. On the addition of lactate oxidase, the shape of the cyclic voltammogram changed dramatically; the reduction peak did not appear and the oxidation peak increased. Figure 2.22. shows cyclic voltammograms before and after the addition of lactate oxidase. The potential window was reduced to 0 – 0.3 V (SCE).

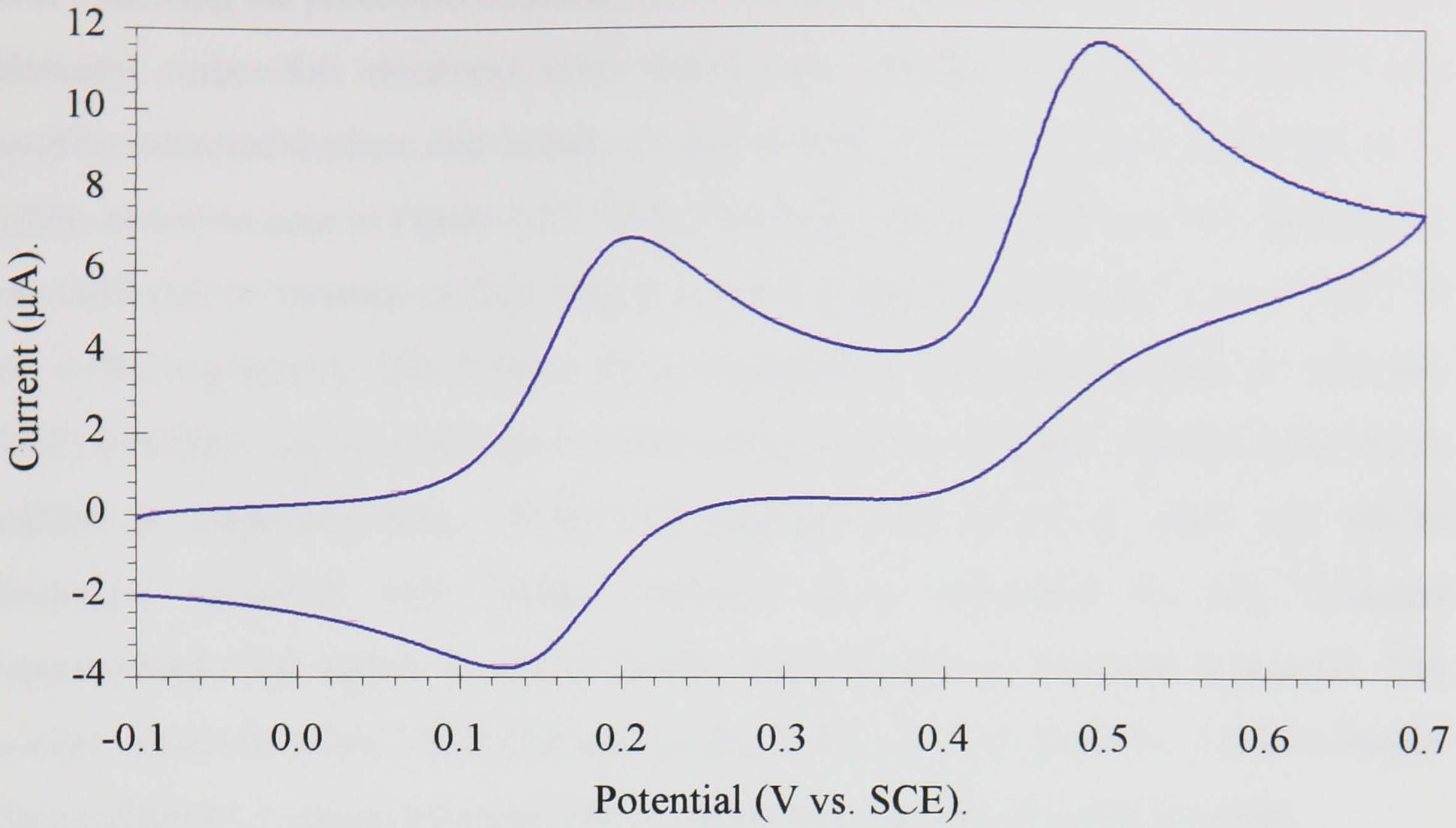


Figure 2.21. Cyclic voltammogram of tetrathiafulvalene in buffer. Platinum working electrode, scan rate 0.05 $V.s^{-1}$ between -0.1 and 0.55 V (SCE).

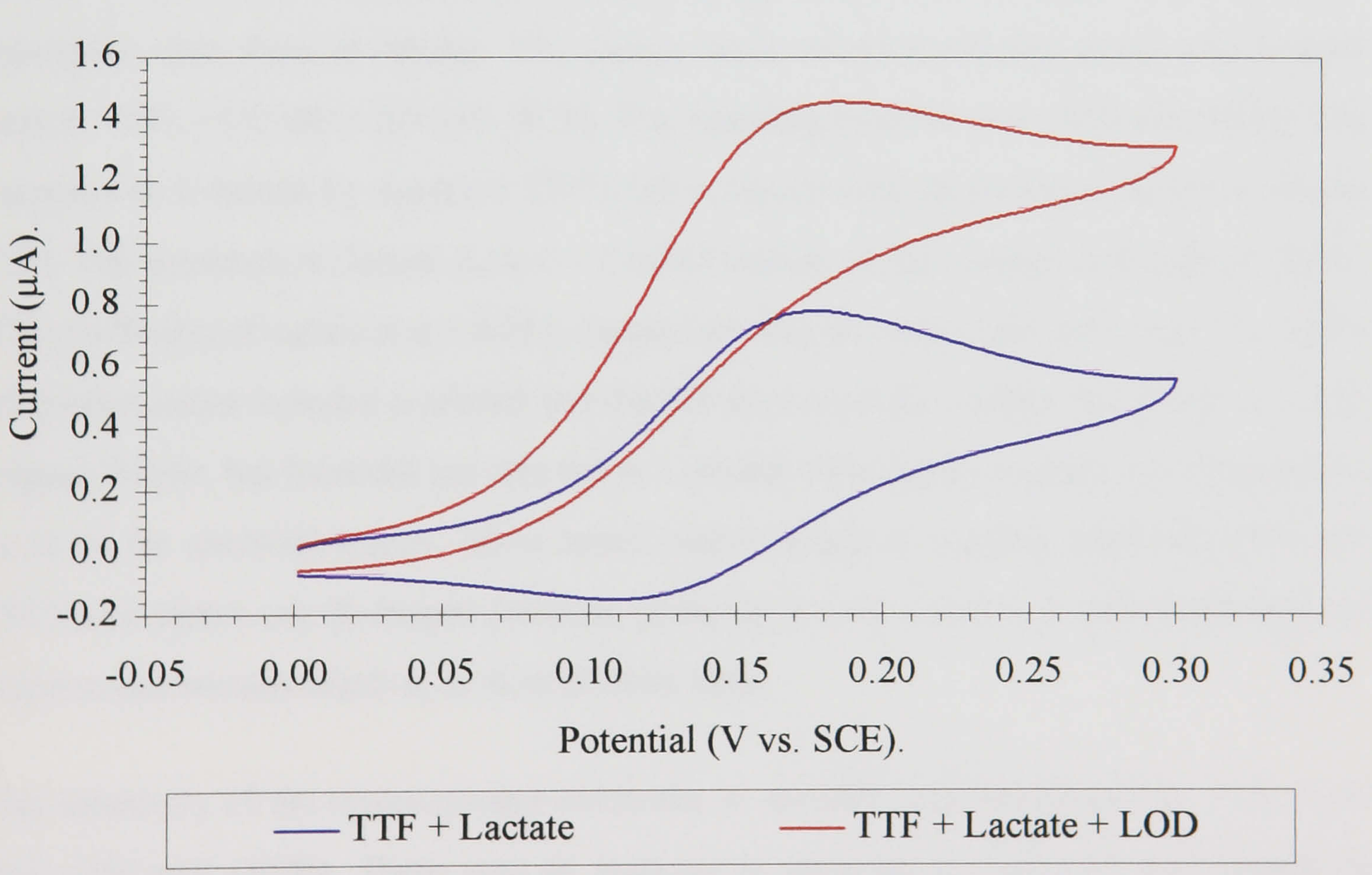


Figure 2.22. Cyclic voltammograms of tetrathiafulvalene with lactate and lactate oxidase in buffer. Potential scanned between 0 and 0.3 V (SCE) at a rate of 0.01 $V.s^{-1}$.

After following the procedure documented by Palleschi and Turner (1990), modified hand-fabricated carbon-foil electrodes were tested with L-lactate at +160 mV (SCE). The resulting tetrathiafulvalene and lactate oxidase sensors (TTF/LOD) responded well to L-lactate, as can be seen in Figure 2.23. There was little drop in response upon repeat testing; the coefficient of variation of four tests at 5 mM L-lactate for electrodes 1 and 2 was 17% and 5.7% respectively. The sensors did not respond to hydrogen peroxide at +160 mV (SCE) and there was no discernible current response from L-lactate without either lactate oxidase or tetrathiafulvalene. When the potential was raised to +800 mV (SCE) electrodes modified with lactate oxidase alone responded to low L-lactate concentrations but above 2 mM L-lactate they elicited no increase in current. The average sensitivity of six TTF/LOD sensors was 0.443 $\mu A.mM^{-1}$ from 0 – 5 mM L-lactate. The coefficient of variation between the six electrodes was 48% at 5 mM L-lactate.

Screen-printed electrodes with a carbon graphite working surface (made with hydroxyethyl cellulose as binder) were heated to 60°C for approximately 24 hours to remove any water from the ink matrix. Tetrathiafulvalene and lactate oxidase were immobilised by simple adsorption onto these electrodes. The sensors were amperometrically tested with L-lactic acid at +100, +150 and +200 mV (SCE). The operating potential was +200 mV (SCE). The response to L-lactate by modified TTF/LOD screen-printed electrodes is shown in Figure 2.24. The sensitivity to lactate from 0 – 2 mM L-lactate of four sensors was 6.92 $\mu A.mM^{-1}$. The coefficient of variation at 4 mM L-lactate between the sensor responses was 42%. Other electrodes tested included a printed tetrathiafulvalene-modified carbon ink or the use of an organic binder, but these did not respond to L-lactate when lactate oxidase was immobilised in or on the electrode matrix. When tested with L-lactate at a higher potential (+800 mV (SCE)) to detect any hydrogen peroxide produced by the enzyme, it was found that the enzyme had become inactive, in most of these cases.

The sensitivity of the screen-printed electrodes to ascorbic acid was tested, at +100, +150 and +200 mV (SCE). There was an increase in response in line with the increase in potential. At an operating potential of +200 mV (SCE) the sensitivity of the TTF/LOD screen-printed electrodes was 26 $\mu A.mM^{-1}$ ascorbate, compared to 20 $\mu A.mM^{-1}$ ascorbate exhibited by unmodified carbon screen-printed electrodes.

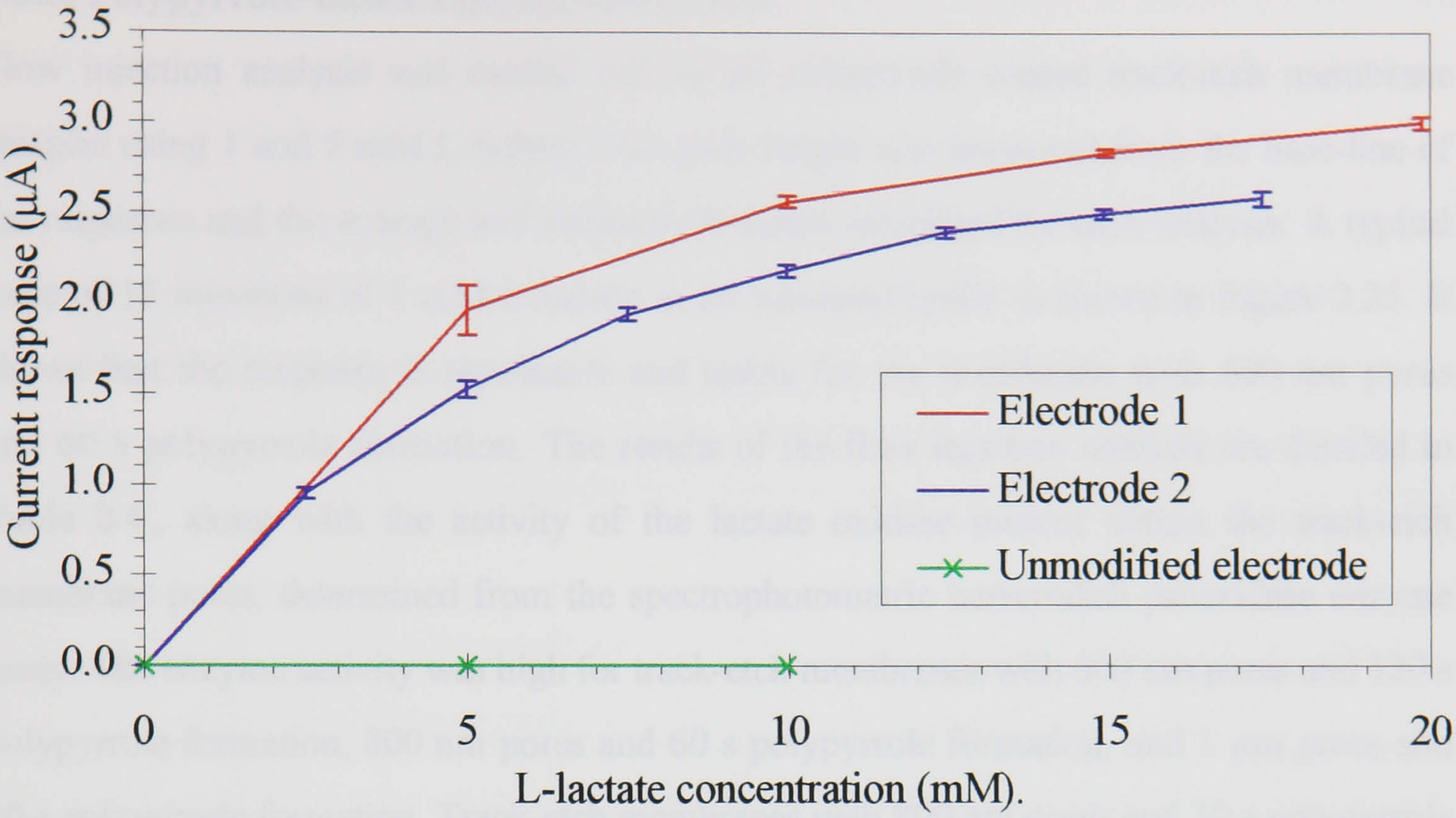


Figure 2.23. Current response of two modified hand-fabricated electrodes to lactic acid at +160 mV (SCE). Electrodes were modified with tetrathiafulvalene and lactate oxidase, four calibrations were carried out in an air atmosphere.

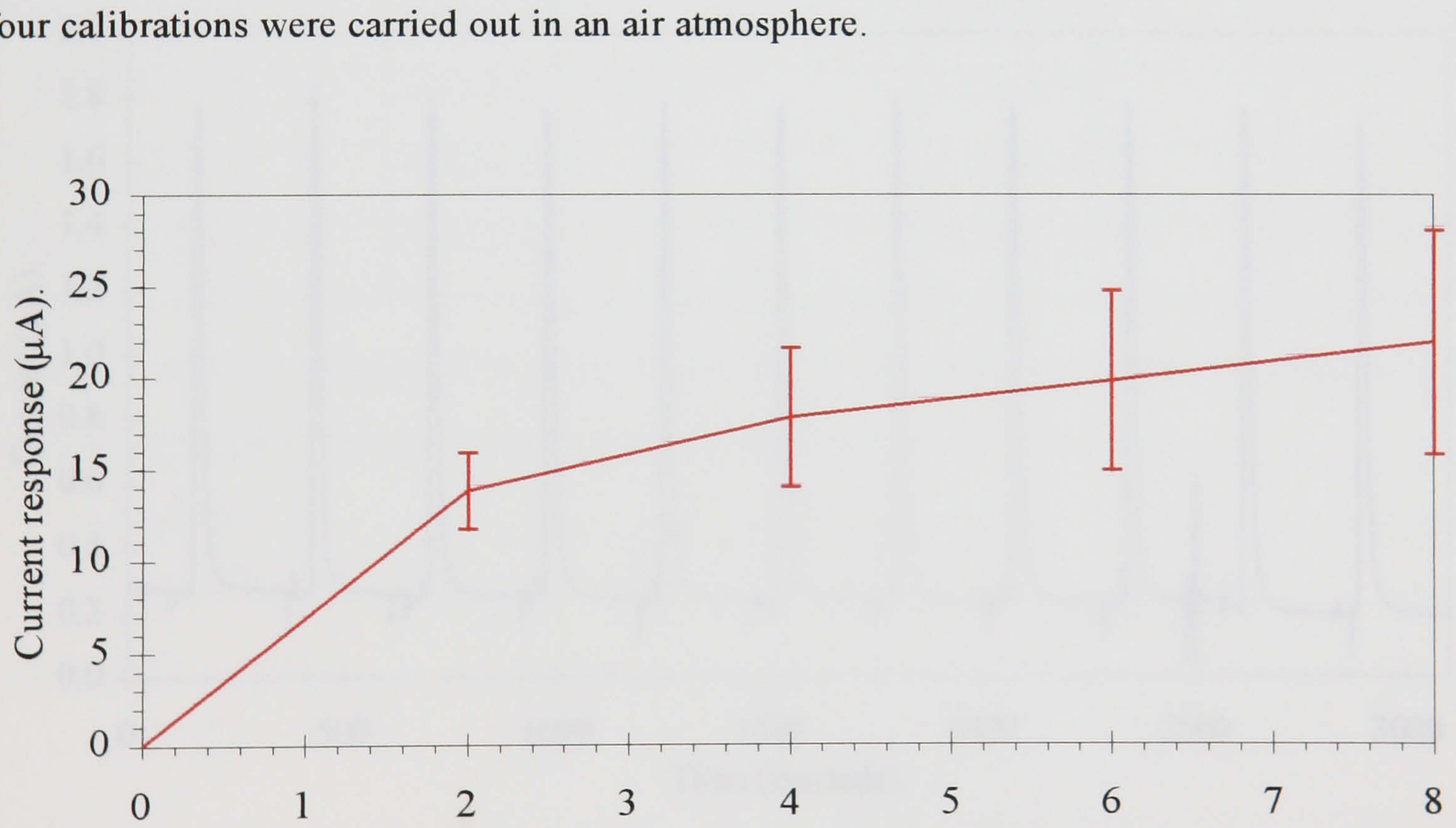


Figure 2.24. Current response to lactic acid at +200 mV (SCE) of modified screen-printed electrodes. Electrodes modified with tetrathiafulvalene and lactate oxidase, four electrode responses.

2.3.5. Polypyrrole-Based Enzyme Electrodes.

Flow injection analysis was carried out on all polypyrrole coated track-etch membrane samples using 1 and 5 mM L-lactate. The peak height was measured from the base-line of ten responses and the average and standard deviation calculated for each analysis. A typical trace of 11 injections of 1 mM L-lactate in air saturated buffer is shown in Figure 2.25. It shows that the response is repeatable and stable for the membrane with 600 nm pores and 60 s polypyrrole formation. The results of the flow injection analysis are detailed in Table 2.4., along with the activity of the lactate oxidase present within the track-etch membrane pores, determined from the spectrophotometric horseradish peroxidase enzyme assay. The enzyme activity was high for track-etch membranes with 600 nm pores and 120 s polypyrrole formation, 800 nm pores and 60 s polypyrrole formation, and 1 μm pores and 30 s polypyrrole formation. Track-etch membranes with 800 nm pores and 30 s polypyrrole formation, and 1 μm pores and 30 s polypyrrole formation gained the highest current responses.

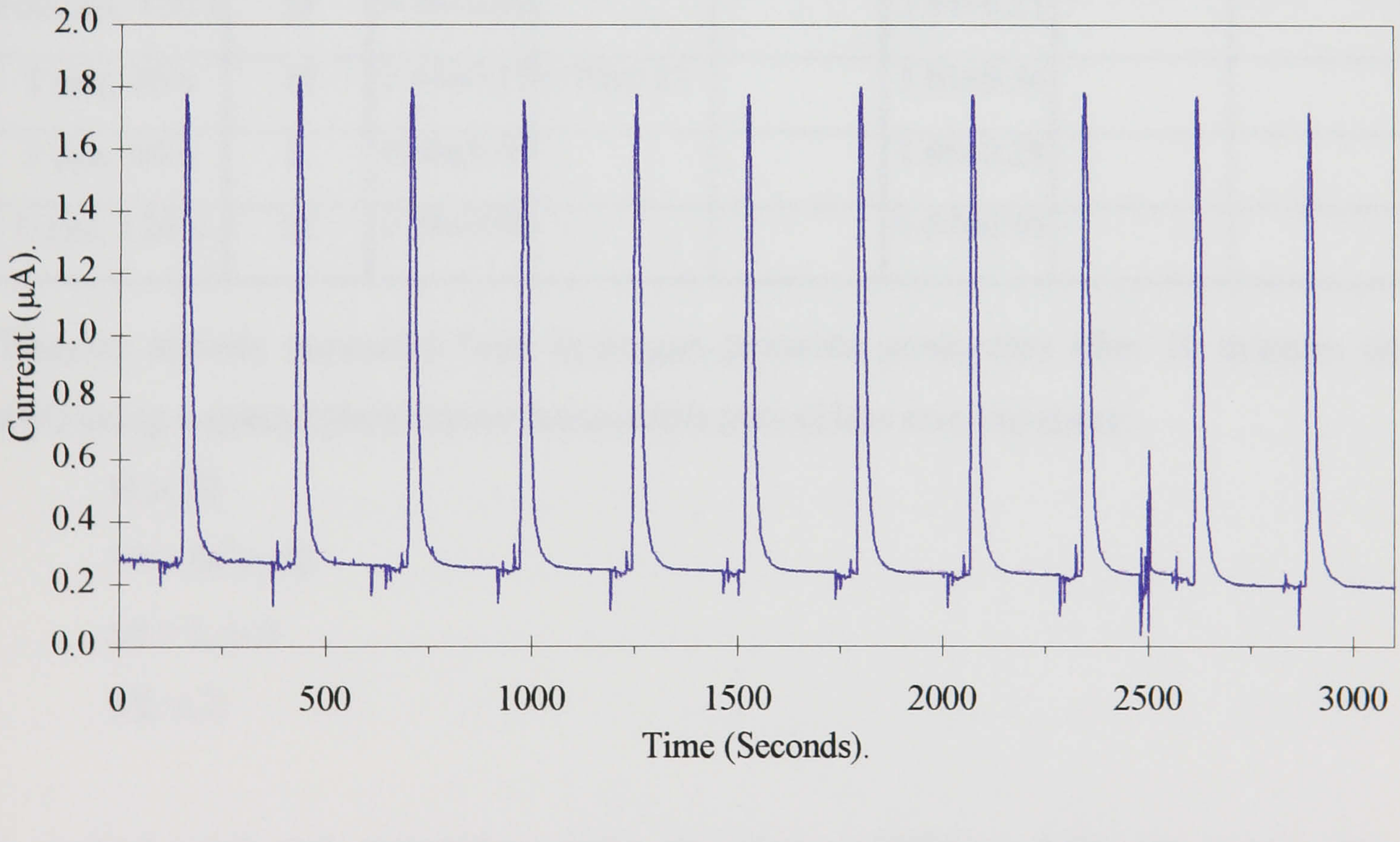


Figure 2.25. Trace of flow injection analysis of L-lactate using track-etch membrane electrodes. Cyclopore membrane of original pore diameter 600 nm with polypyrrole formation for 60 s. Flow rate 0.5 $ml.min^{-1}$ with 1 mM L-lactate injections every 4 minutes. Buffer and L-lactate solution contained peroxidase. Potential held at +350 mV (Ag/AgCl).

Table 2.4. Summary of enzyme activity and flow injection responses to lactate by track-etch membranes incorporating polypyrrole and lactate oxidase. Current response (in μA above baseline) of 10 injections plus and minus the standard deviation.

Track-etch membrane	Enzyme activity[#]	1 mM lactate injections in air on different sections of membrane.			5 mM lactate injections in air on different sections of membrane.		
		First	Second	Third	First	Second	Third
Control, 600 nm 30 s	VL	0.33±0.02			0.10±0.02		
600 nm, 30 s	M	0.73±0.06	1.29±0.12	1.76±0.18	0.33±0.02	2.95±0.52	5.37±0.24
600 nm, 60 s	L	0.53±0.03	1.54±0.02		1.90±0.14		
600 nm, 120 s	H	0.56±0.02	0.66±0.06		2.1± 0.16		
800 nm, 30 s	L	3.59±0.12			8.98±0.77		
800 nm, 60 s	H	0.81±0.03	0.83±0.08		3.28±0.18		
800 nm, 120 s	M	0.56±0.02			2.84±0.21		
1 μm, 30 s	H	2.44±0.13	1.70±0.23		5.03±0.36		
1 μm, 60 s	L	0.60±0.03			1.86±0.29		
1 μm, 120 s	M	0.58±0.09			1.07±0.09		

[#]Enzyme activity measured from hydrogen peroxide production after 10 minutes (in mM) using a spectrophotometric horseradish peroxidase enzyme assay:-

$H \geq 70$

$70 > M \geq 50$

$50 > L \geq 2$

$VL \leq 2$

A control track-etch membrane under the same conditions (600 nm pores, 30 s polypyrrole formation, no lactate oxidase) with 1 mM hydrogen peroxide gave a current response of approximately 4.8 μA. Hydrogen peroxide (1 mM) on the bare glassy carbon electrode under the same conditions gave no distinguishable current response.

Oxygen, unfortunately, could not be excluded from the system because the tubing used to and from the pumps on the flow injection system were gas permeable. Even though nitrogen was bubbled through the solutions, due to the slow flow rate, oxygen could enter back into the solutions before they entered the cell. Under reduced oxygen conditions there was a general trend of 30% fall in current response.

When peroxidase was added to the buffer (to react with any hydrogen peroxide), the response was the same as that of the reduced oxygen conditions response on both the 600 nm pore, 30 s polymerisation track-etch membranes with and without lactate oxidase.

A brief investigation into the method of transduction was undertaken using cyclic and square wave voltammetry. Unfortunately, no peaks were visible with cyclic voltammetry and only a small oxidation shoulder between -700 and -350 mV (SCE) was visible with square wave voltammetry, only when the potential started below -650 mV (SCE) with a step frequency of at least 20 Hz.

Screen-printed electrodes modified with the latex/polypyrrole/lactate oxidase ink were tested at +350 mV (SCE) in buffer solution with L-lactate and hydrogen peroxide. Four electrodes were tested, under both air and nitrogen atmospheres. Their responses varied greatly but followed the trend as outlined in Table 2.5. A first response to L-lactate was gained under a nitrogen atmosphere but any further additions of L-lactate did not increase the current. Further tests under a nitrogen atmosphere elicited smaller responses. Calibrations under air atmospheres produced a very short detection range with currents only slightly larger than under a nitrogen atmosphere. The response to hydrogen peroxide was greater under an air atmosphere than under a nitrogen atmosphere but currents to the equivalent concentration of hydrogen peroxide to L-lactate gave smaller responses. The dynamic range of hydrogen peroxide detection was much larger than that of L-lactate.

Table 2.5. Typical current responses to L-lactate and hydrogen peroxide under an air or nitrogen (N_2) atmosphere of latex/lactate oxidase-modified screen-printed electrodes. Potential held at +350 mV (SCE). The detection limit for this electrode was 0.5 mM lactate (considering a signal to noise ratio of 3) and the upper limit of detection was 1.5 mM lactate.

L-lactate concentration	Current response (in μA).				H_2O_2 concentration	Current response (in μA).	
(mM)	N_2	Air	N_2	Air	(mM)	N_2	Air
0.5	0.097	0.046	0	0.026	0.5	0.016	0.023
1.0	0.103	0.095	0	0.039	1.0	0.042	0.049
1.5	0.099	0.098	0	0.041	1.5	0.065	0.075
2.0	0.099	0.098			2.0	0.091	0.103
2.5	0.097	0.105			2.5	0.117	0.130

2.3.6. Electrochemical Surface Area.

A typical chronocoulometric plot obtained from a platinum disc electrode in hexacyanoferrate (II) solution is shown in Figure 2.26. and the corresponding charge versus square root of time is shown in Figure 2.27. All chronocoulometric Q vs. $t^{1/2}$ plots were very linear when measured on a time scale shorter than 1s (with correlation coefficient $r^2 \geq 0.998$), above this they began to level off. The intercept did not pass through the origin but cut through the x-axis. The slope of the line gave the electrochemical surface area of the electrode, each electrode had a slightly different slope but electrodes of the same type gave very similar values.

The surface areas are detailed in Table 2.6., comparing geometric with electrochemical values. There is a good correlation between the geometric and electrochemical surface area for the platinum disc electrode and only a small error between measurements. The screen-printed electrodes gave very small errors whereas the hand fabricated electrodes showed greater variability.

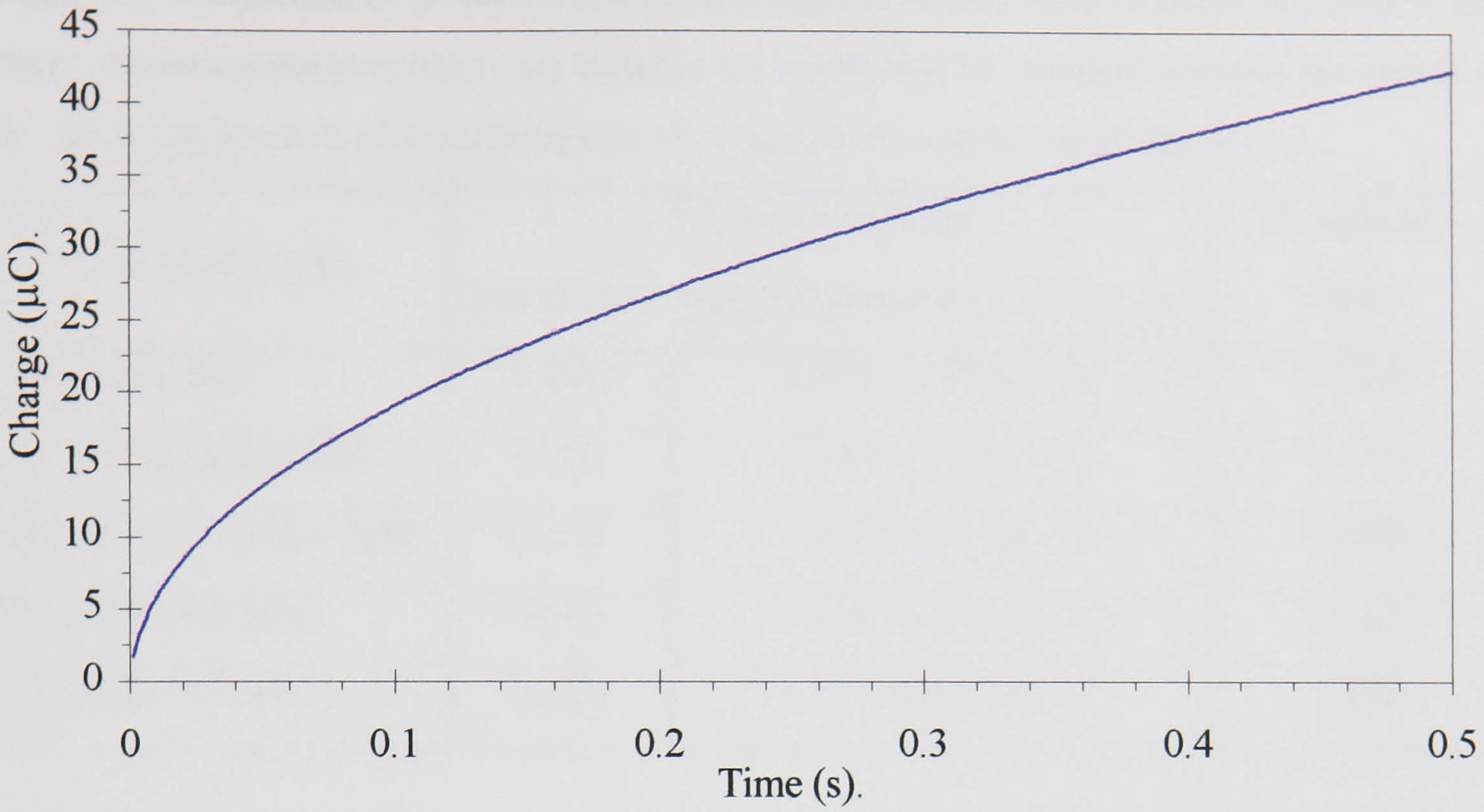


Figure 2.26. Chronocoulometric response of a platinum disc electrode in hexacyanoferrate (II) solution, upon the application of +450 mV (SCE).

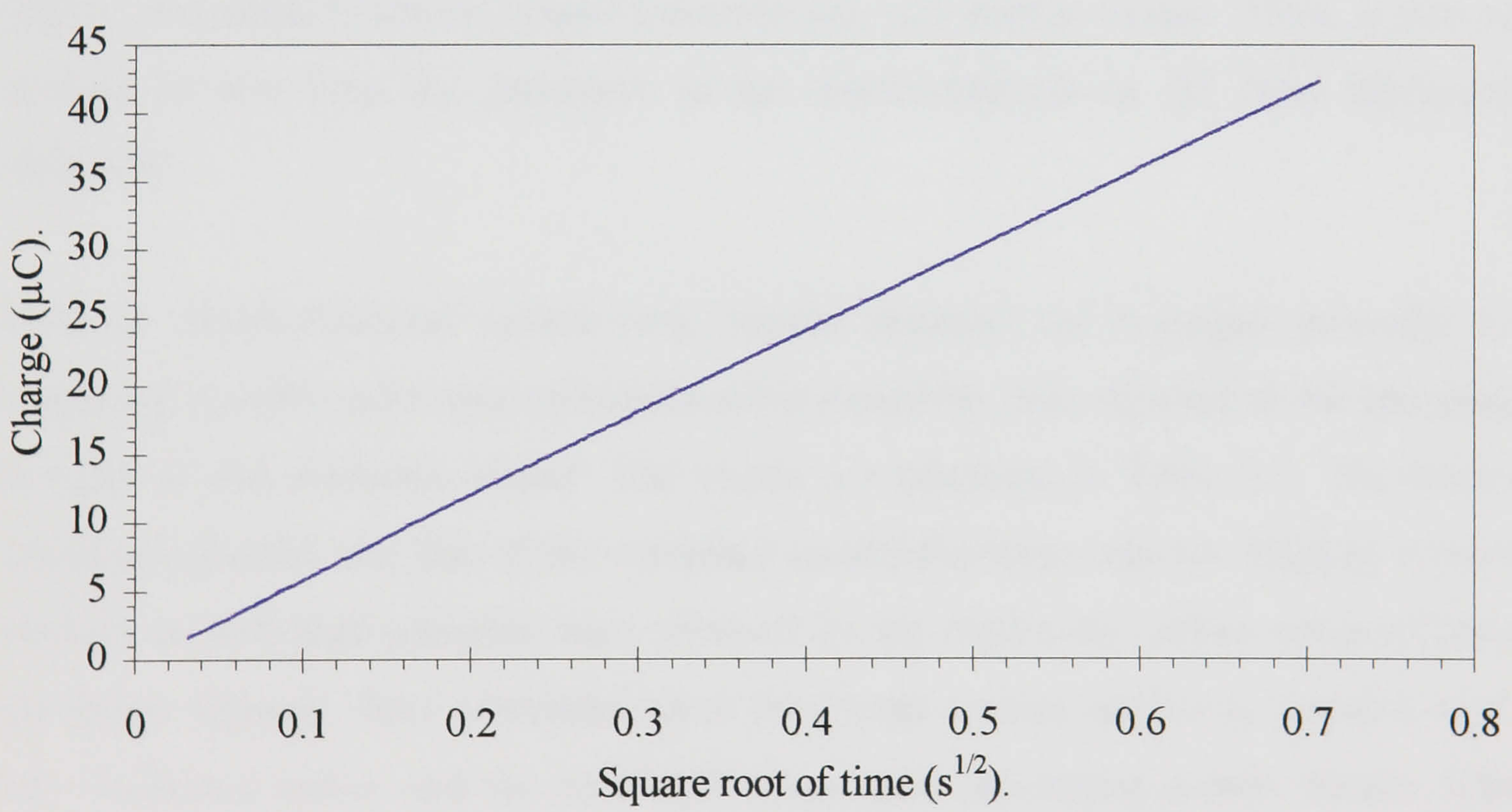


Figure 2.27. Anson plot from the platinum disc electrode in hexacyanoferrate (II) solution, upon the application of +450 mV (SCE).

Table 2.6. Comparison of geometric and electrochemical surface areas of electrodes used in this study. Electrochemical surface area includes the coefficient of variation between the slopes of the chronocoulometric plots with number of comparisons in next pair of parenthesis.

Electrode Type	Surface area (cm^2)		Roughness factor
	Geometric	Electrochemical (C.V.%), {n}	
Platinum disc	0.020	0.022 (2.1%) {10}	1.108
Carbon graphite SPE	0.151	0.199 (3.7%) {4}	1.324
Rhodinised carbon SPE	0.152	1.010 (5.3%) {5}	6.648
Graphite rod HFE	0.283	0.744 (15.3%) {5}	2.632
Graphite foil HFE	0.679	1.277 (28.3%) {5}	1.880

The roughness factor of the electrodes was calculated as the ratio of electrochemical surface area to geometric. It can be seen that there is a large difference between the electrochemical and geometric area of the rhodinised-carbon screen-printed electrode whereas the graphite screen-printed electrode has very similar values. There is also an increase in area from the geometric to the electrochemical on the hand fabricated electrodes.

From the electrochemical surface area, current densities for hydrogen peroxide, L-lactate and ascorbic acid were calculated using sensitivity data obtained at the operating potential of the particular sensor. The results are tabulated in Table 2.7. The lowest operating potential was that of the mediated tetrathiafulvalene sensors. Highest current densities to hydrogen peroxide were obtained by the rhodinised carbon screen-printed electrodes although these electrodes gave the lowest current density to ascorbic acid. The rhodinised carbon and the tetrathiafulvalene gave the largest current density with L-lactate in the presence of lactate oxidase. The highest interference from ascorbic acid was seen on the platinum disc electrode. It is interesting to note that although the operating potential of the tetrathiafulvalene sensors was much lower than that of the hexacynaoferrate (III)/ lactate oxidase sensors, they gave a higher response with ascorbic acid. Upon the immobilisation of lactate oxidase, the modified-carbon electrodes all responded to lactate. The linear and dynamic ranges, response time and

repeatability of the sensors varied and Table 2.7 illustrates the difference in sensitivity. The rhodinised carbon and tetrathiafulvalene-modified carbon showed the greatest current density with L-lactate but it must be remembered that there was different enzyme loading on the sensors (1.8 U per rhodinised carbon screen-printed electrode and 9 U per tetrathiafulvalene modified carbon screen-printed electrode, measured by weight).

Table 2.7. Electrode current density with hydrogen peroxide, lactic acid and ascorbate at a particular potential. Current density measured as current gained per unit concentration of analyte per electrochemical surface area ($\mu A.mM^{-1}.cm^{-2}$). x = not tested.

Electrode Type	Potential (mV vs. SCE)	Hydrogen peroxide	L-lactic acid	Ascorbic acid
Platinum disc	+500 / +400	62 / 53	x	417 / 321
Hex.LOD HFE	+500	1.1	1.5	118
Hex.LOD SPE	+500	3.1	x	98
RhC.LOD SPE	+400	27	6.8	26
TTF.LOD HFE	+160	x	0.35	x
TTF.LOD SPE	+200	x	35	131

2.4. DISCUSSION.

2.4.1. Electrochemical Characteristics of Hexacyanoferrate (III)-modified Carbon Electrodes.

The cyclic voltammograms obtained during and after the modification of carbon electrodes with hexacyanoferrate (III) are in very good agreement with those obtained by Gomathi and Prabhakara Rao (1990) and Jaffari (1994 and Jaffari & Turner, 1997). The peak observed during the second scan of modification is consistent with Fe (II) oxidising to Fe (III) (Jaffari, 1994; Jaffari & Turner, 1997). The two peaks which increased with time and prominently seen on the fifteen scan (Figure 2.5.), are consistent with those observed by Jaffari (1994), indicating film formation. A green solution with green precipitate obtained during the modification was also indicative of film formation and could be attributed to the presence of Berlin Green. Berline Green is the oxidised form of Prussian Blue (iron hexacyanoferrate) and was probably formed by the combination of hexacyanoferrate (II) with liberated iron (III) during the electrode modification. This theory is supported by Gomathi and Prabhakara Rao (1990).

By investigating the electrochemistry of rinsed electrodes in potassium chloride solution and comparing the results with previous reports (Gomathi & Prabhakara Rao, 1990; Jaffari, 1994), it was determined that the carbon electrodes were modified with hexacyanoferrate (III). The cyclic voltammogram of a hexacyanoferrate-modified electrode in Figure 2.6. show some interesting features. The redox peaks occurring at approximately +200 mV (SCE) had a peak separation much less than 59 mV and a sharp and narrow appearance which are indicative of adsorbed electro-active species (Bard & Faulkner, 1980). The potential is consistent with the potassium hexacyanoferrate redox couple, suggesting that this had been adsorbed into the carbon matrix. The redox peaks occurring at approximately +900 mV (SCE) are smaller and less sharp but have a peak separation less than 59 mV, suggesting another electro-active species was adsorbed on the electrode. Cyclic voltammograms of a Prussian Blue-modified glassy carbon and platinum electrodes in potassium chloride solution, reported by Itaya *et al.* (1984) and Viehbeck (1985) respectively, showed two sets of redox peaks at +200 and +900 mV (SCE). These peaks were attributed to the three main valence forms of the film: Prussian White (Everitt's salt)

for E<0.2 V, Prussian Blue for 0.2<E<0.9 V and Berlin Green for E>0.9 V (Viehbeck & DeBerry, 1985). Gomathi and Prabhakara Rao modified a glassy carbon electrode with hexacyanoferrate and observed similar cyclic voltammetric responses in potassium chloride. They noted that the modification may have resulted in the formation of Prussian Blue on the electrode surface which would manifest as the reversible peak to Berlin Green around +900 mV (SCE). This evidence leads to the hypothesis that not only has potassium hexacyanoferrate been adsorbed onto the carbon, but that Prussian Blue has been formed, itself adsorbing onto the carbon and exhibiting redox peaks at +200 and +900 mV (SCE).

On the addition of L-lactate and lactate oxidase, no catalytic response was observed in the cyclic voltammograms. This indicates that there was no mediation of the enzyme by the hexacyanoferrate (III)-modified electrode which is consistent with results obtained with glucose oxidase and hexacyanoferrate (III)-modified electrodes by Jaffari (1994).

The work by Jaffari (1994 and 1997) was taken further, by modifying screen-printed carbon graphite electrodes. Electrodes produced with carbon ink made with cellulose acetate in acetone were modified. Phenolic and/or carboxylic groups generated by oxidation above +1.5 V (SCE) create localised acidity which is associated with modification with hexacyanoferrate (Gomathi & Prabhakara Rao, 1990). Perhaps attempts at modification of other ink formulations did not succeed because the localised acidification did not occur in such matrixes.

The electro-catalytic behaviour of hexacyanoferrate (III)-modified carbon electrodes was observed by carrying out hydrodynamic voltammograms. This also allowed the optimal operating potential to be chosen. Slightly different behaviour was observed on the graphite rods compared to the screen-printed electrodes, possibly due to the different physical and chemical nature of the electrodes.

In order to evaluate the possibility of oxygen being detected by the hexacyanoferrate (III)-modified electrodes, experiments were also carried out under a nitrogen atmosphere. Oxygen was reduced at the electrode at potentials below +200 mV (SCE). Reduction of oxygen at Prussian Blue-modified glassy carbon electrodes occurs at potentials below

+175 mV (SCE) (Itaya *et al.*, 1984; Karyakin *et al.*, 1994a). It was decided not to detect the reduction of hydrogen peroxide since variations in the ambient oxygen concentration would affect the measurement. Instead, oxidation of hydrogen peroxide at +500 mV (SCE) was the chosen detection method, since an increased current was observed at this potential on both hand fabricated and screen-printed electrodes in comparison to the unmodified electrodes. Jaffari (1994; 1997) used an operating potential of +450 mV (SCE) although in other work preferred +750 mV (Ag/AgCl) since a larger current response was gained (Jaffari & Pickup, 1996).

By increasing the number of scans during modification, the electro-catalytic behaviour towards hydrogen peroxide was increased. This was not observed by Jaffari who found that increased modification time actually decreased the sensitivity to hydrogen peroxide (Jaffari, 1994; Jaffari & Pickup, 1996).

On repeated amperometric testing of the hexacyanoferrate (III)-modified electrodes with hydrogen peroxide, it was noted that the current response decreased. This trend was more exaggerated for the modified screen-printed electrodes, especially for those modified with a greater number of scans. In addition, the response to ascorbic acid increased with time. This suggests that the electrode modification changed with testing, perhaps the adsorbed hexacyanoferrate dissociated or the film began to break away. This may also explain the behaviour of the electrodes with increased modification - more hexacyanoferrate (III) was adsorbed and not more film created.

Unfortunately, the screen-printed electrodes gave very different sensitivities to hydrogen peroxide although they should had very similar surface areas. This meant that although they were modified with the same number of scans, they did not show modification to the same degree.

The ascorbic acid response of hexacyanoferrate (III)-modified and unmodified electrodes was further characterised at a range of potentials in this work. The response to ascorbic acid was greater on the platinum disc electrode than on the carbon electrode. Additionally, the hexacyanoferrate (III)-modified carbon electrodes gave a greater response to ascorbic acid at lower potentials, but a lower response at higher potentials than the unmodified carbon

electrodes. Prussian Blue films have been reported to catalyse the oxidation of ascorbic acid effectively (Dong & Che, 1991), however, Jaffari (Jaffari, 1994; Jaffari & Pickup, 1996; Jaffari & Turner, 1997) observed less of a response from ascorbic acid on modified compared to unmodified electrodes. The results obtained here support the hypothesis that the hexacyanoferrate (III)-modification may contain some Prussian Blue, but performance does differ from the 'pure' PB films.

This is the first report of lactate measurement with the use of lactate oxidase immobilised on hexacyanoferrate (III)-modified electrodes. It has been shown by Jaffari (Jaffari, 1994; Jaffari & Pickup, 1996; Jaffari & Turner, 1997) that glucose oxidase immobilised on hexacyanoferrate (III)-modified electrodes can detect glucose concentrations. The principle was tested on modified carbon-rod electrodes in this work since they were more stable. The lactate sensors gave a fast response to lactate over a wide range, even without the use of membranes. These response characteristics were achieved by the use of porous carbon-graphite rod electrodes which produce a hydrodynamic restriction to the diffusion of L-lactate (Cardosi & Turner, 1987).

2.4.2. Electro-catalytic Behaviour of Prussian Blue-modified Graphite Screen-Printed Electrodes.

Since Prussian Blue (PB) is commercially available as a powder, it is amenable to carbon ink formation and the screen printing process. Prussian Blue-modified carbon screen-printed electrodes were tested to check if the response was similar to the hexacyanoferrate (III)-modified electrodes so that the complete electrode could be fabricated by the screen printing process.

The hydrodynamic voltammograms were carried out in order to observe the electro-catalytic behaviour of hydrogen peroxide with PB screen-printed electrodes and to enable the optimal operating potential to be chosen. The results are in good agreement with reports of hydrogen peroxide detection by PB films on glassy carbon electrodes (Itaya *et al.*, 1984; Karyakin *et al.*, 1995).

The first operating potential chosen was at +600 mV (SCE) because it catalytically oxidised hydrogen peroxide with a detectable current and the amount of interference from biological species would be lower than detecting at a higher potential. However, the response to hydrogen peroxide at this potential was much less than that obtained at hexacyanoferrate (III)-modified electrodes. It was found that the greater the PB content, the greater the electro-catalysis, agreeing with work carried out by Itaya *et al.* (Itaya *et al.*, 1984) using PB films on glassy carbon electrodes and by Boyer *et al.* (Boyer *et al.*, 1990) using PB on carbon paste electrodes. The response to hydrogen peroxide did not increase proportionally with PB content, instead it increased less. From this it can be assumed that even a higher PB content will not elicit as great a response to hydrogen peroxide as the hexacyanoferrate (III)-modified carbon electrodes.

Zero millivolts (SCE) was investigated as an operating potential because greater current responses were observed and it was thought that lower potentials would elicit less response from interferents. There was neither intra- or inter- electrode repeatability at 0 mV (SCE), probably due to a combination of interference from oxygen and instability of the modified carbon ink. This potential has been found by Itaya *et al.* (1984) and Karyakin *et al.* (1995) to be optimal for hydrogen peroxide reduction, but they showed that oxygen was catalytically reduced at this potential (Itaya *et al.*, , 1984; Karyakin *et al.*, 1995). The soluble form of PB ($KFe\{Fe(CN)_6\}$), found in commercial dyes, can leech out (Boyer *et al.*, 1990), leading to electrode instability.

The sensitivity to ascorbic acid increased with PB modification in comparison to the unmodified carbon electrode as can be seen from the results at 0 mV (SCE). This agrees with results published by Dong and Che (1991) who observed that ascorbic acid is electro-catalytically oxidised at PB film-modified platinum electrodes.

2.4.3. Electro-catalytic Properties of Rhodinised-Carbon Screen-Printed Electrodes.

Rhodinised carbon was used as an alternative to platinised carbon since there were reports of its good electro-catalytic behaviour towards hydrogen peroxide when used as a

transducer for an amperometric glucose sensing device (Wang & Angnes, 1992; White, 1993) and lactate sensing device (White, 1993).

The hydrodynamic voltammograms of rhodinised carbon screen-printed electrodes tested with hydrogen peroxide obtained in this work is in good agreement with results obtained of rhodinised carbon-rod electrodes (White, 1993). The rhodinised surface of the carbon provided catalytic sites which significantly decreased the over potential required to detect the hydrogen peroxide with oxidation occurring above +190 mV (SCE) and reduction below. The plateau in the response above +400 mV (SCE) obtained in this study can be seen more clearly at higher hydrogen peroxide concentrations. By choosing the operating potential at the start of the plateau response, any small variation in potential will not adversely affect the current response. The hydrodynamic voltammograms obtained by Wang and Chen (1994) with rhodinised-carbon glucose-oxidase modified screen-printed micro-disc array electrodes tested with glucose showed a similar plateau response starting at approximately +450 mV (Ag/AgCl). The inter- and intra- electrode reproducibility was better than the hexacyanoferrate (III)-modified screen-printed electrodes, although the linear range was shorter.

At +400 mV (SCE), the interference from ascorbic acid was less at the rhodinised carbon than at the unmodified carbon-graphite screen-printed electrodes, but was more than that observed at hexacyanoferrate (III)-modified screen-printed electrodes. The hydrogen peroxide to ascorbic acid ratio of sensitivity in this work was approximately 1, whereas White (1993) observed a ratio of approximately 3. This cannot be fully explained, but could be due to a variation in the rhodium content since the particle size of the metal influences the catalytic behaviour of the modified carbon (Mukerjee, 1990).

There have been no reports of lactate oxidase being printed onto rhodinised carbon screen-printed electrodes, although lactate oxidase screen-printed onto metallised electrodes has recently been reported (Collier *et al.*, 1996; Hart *et al.*, 1996; Rohm *et al.*, 1996). Previously, lactate oxidase was either electrochemically co-deposited with rhodium onto screen-printed carbon arrays (Wang & Chen, 1994), cast in a membrane on the electrode surface (White, 1993; White *et al.*, 1994a; White *et al.*, 1994b) or immobilised within a

rhodium dispersed carbon paste (Wang *et al.*, 1995a). Fabrication of the screen printed lactate oxidase/rhodinised-carbon electrodes will be discussed later in this thesis.

The lactate oxidase/rhodinised-carbon screen-printed electrodes gave very good intra- and inter- electrode stability, high sensitivity to lactate and a fast response. Although the dynamic range to lactate of these sensors was not as great as the lactate oxidase/hexacyanoferrate (III)-modified carbon rod sensors, it could be extended by applying diffusion limiting membranes (see Chapter 4).

These results show that this fabrication method is very suitable for manufacturing stable and sensitive enzyme electrodes with a high degree of reproducibility.

2.4.4. Electrochemical Behaviour of Tetrathiafulvalene-modified Carbon Electrodes.

Tetrathiafulvalene has extremely low solubility in aqueous solutions (about 1 $mg.L^{-1}$) (Zhao & Luong, 1993) and so incorporation into a water-soluble complex to form a micelle type suspension has been used to observe its electrochemical behaviour. A detergent (Tween 80) was used to dissolve the tetrathiafulvalene before buffer was added. The resulting solution was de-aerated with the exclusion of light during storage and experiments, to keep the tetrathiafulvalene active. This method of pseudo-dissolving tetrathiafulvalene has also been used by Hendry (1989) to observe the catalytic wave effect of glucose oxidase on tetrathiafulvalene during linear sweep voltammetry. A cyclic and non-reducing, water-soluble oligosaccharide (2-hydroxypropyl-β-cyclodextrin) was used by Zhao and Luong (1993) to enclose tetrathiafulvalene and form a water-soluble complex with which catalytic waves using cyclic voltammetry were observed with glucose oxidase, zanthine oxidase and lactate oxidase. They reported that this tetrathiafulvalene solution in buffer remained active and unaffected by oxygen in solution if kept at 4°C in the dark, for four weeks.

The two oxidation peaks in Figure 2.21. relate to the two, one electron processes of tetrathiafulvalene, that is, its oxidation to the radical cation and the di-cation (Bryce, 1985; Jaeger & Bard, 1979). The first oxidation peak also has a corresponding reduction peak, whereas the second oxidation peak does not have a defined reduction peak. The first oxidation process is reversible whereas the oxidation to the di-cation is irreversible and it

is the first process which is involved in the electron transfer mediation process with oxidoreductase enzymes (Lee *et al.*, 1992; Palleschi & Turner, 1990; Zhao & Luong, 1993).

There is a slight shift of the cyclic voltammogram to more positive potentials once lactate has been added, as well as an increase in peak height. The peak shift may be due to the presence of the lithium from the lactic acid preparation since it is has been reported that the potentials for the oxidation and reduction peaks are different in different supporting electrolytes (Jaeger & Bard, 1979; Liu *et al.*, 1995). Lithium also forms a salt with tetrathiafulvalene which is more soluble than the chloride salt (Jaeger & Bard, 1979) and this may explain the peak height increase. When lactate oxidase was added, the oxidation peak increased, however, the reduction peak was absent. This demonstrates that the tetrathiafulvalene mediates the lactate oxidase catalysis of lactate to pyruvate in the manner previously described (see Section 2.1.). This is consistent with reports by Zhao and Luong (1993) and Liu (1995).

Preliminary experiments were carried out using the modified carbon-foil electrodes to observe the coupling between tetrathiafulvalene and lactate oxidase under an air atmosphere. The electrodes responded well to L-lactate and gave repeatable responses. It has previously been reported by Palleschi and Turner (1990), and White *et al.* (1992) that repeat testing resulted in a decrease in response to L-lactate and was thought to be due to loss of enzyme. Perhaps in this study, the enzyme was more strongly bound due to a more thorough drying process. Surface-oxidised functional groups on carbon electrodes impart considerable hydrophilicity and ionic character (Frew & Hill, 1988) which aids protein binding. However, it is believed that water competes for these sites. Koopal (1992) found that drying after enzyme immobilisation was essential for good association.

Screen printing tetrathiafulvalene and lactate oxidase was not at first successful. Both lactate oxidase and tetrathiafulvalene are known to be unstable and it is assumed that the harsh conditions of the printing process deactivated one or both compounds. Tetrathiafulvalene has been successfully screen printed previously by Bilitewski *et al.* (1992; 1993), but was used in conjunction with glucose oxidase, a stable enzyme (Wilson & Turner, 1992). The report by Bilitewski *et al.*, together with the lack of hydrogen peroxide detection at +800 mV

(SCE) would suggest that lactate oxidase denaturation occurred in this study. However, if carbon electrodes were first printed and then aliquots of tetrathiafulvalene and lactate oxidase were added to the surface, under controlled temperature and atmospheric conditions, the activity was retained. The response to L-lactate by the screen-printed electrodes followed a similar trend to the modified carbon-foil electrodes, but the current response was much greater. This could be due to higher tetrathiafulvalene and lactate oxidase concentrations resulting at the electrode surface from applying a volume of solution and allowing the liquid to evaporate rather than relying on diffusion and adsorption from bulk solution. The reproducibility of the sensors was not as good as desired, but this could be improved by controlling the drop deposition of the tetrathiafulvalene and lactate oxidase solutions by automation (e.g., by using an ink-jet printer or drop-on-demand device such as a Cavro printer).

Different atmospheres were not studied since this was a preliminary examination of the mediation concept for a biosensor to be used in oxygenated biological solutions. It is already known that responses from low L-lactate concentrations in oxygenated conditions are not linear due to competition between the natural and artificial electron acceptors (Palleschi & Turner, 1990). Therefore L-lactate concentrations below 2 mM were not tested.

Even though the operating potential of the tetrathiafulvalene and lactate oxidase-modified electrodes was lowered to +200 mV (SCE), there was significant current obtained from ascorbic acid oxidation. Interference from ascorbic acid is known to be troublesome on carbon electrodes, even at low potentials (Gorton *et al.*, 1991; Kulys *et al.*, 1992a), but it can be limited by the application of membranes (Vadgama, 1990).

The benefits of tetrathiafulvalene as a mediator are that it reacts rapidly with the reduced enzyme, exhibits reversible heterogeneous kinetics for the first oxidation state and is stable in the first oxidised and reduced state, and has a low mediating potential. However, tetrathiafulvalene is a toxic compound which shows a variation in response in different buffers as well as being unstable in air, and can be irreversibly over-oxidised. Although tetrathiafulvalene does fulfil some criteria needed for a practical mediated enzyme electrode outlined by Cardosi and Turner (Cardosi & Turner, 1987) it has some inadequacies. These

inadequacies would limit its use to an *ex-vivo* where only one measurement should be made with each device to be assured of a reliable estimate.

2.4.5. Performance of Lactate Oxidase with Polypyrrole.

Track-etch membranes incorporating polypyrrole and lactate oxidase have not previously been reported.

Flow injection analysis was used in the preliminary study of lactate oxidase and polypyrrole behaviour because the membrane sections were not amenable to conventional electrode fabrication which would have allowed batch measurements to be carried out. The flow conditions were chosen on the basis of achieving a repeatable and large peak height with a stable background current.

The bare glassy carbon electrode used in this study did not detect hydrogen peroxide at +350 mV (Ag/AgCl), but when a track-etch membrane (with a sputtered platinum face) was placed against the electrode, the oxidation of hydrogen peroxide was detected. This indicates that any current responses could have been gained from the oxidation of enyzmically produced hydrogen peroxide. Although Koopal (1992) detected hydrogen peroxide reduction at 0.1 V (Ag/AgCl), no report was made of detection at +350 mV (Ag/AgCl).

It is postulated that hydrogen peroxide oxidises the polypyrrole film quite rapidly and causes it to loose its electrical conductivity (Belanger *et al.*, 1989; Kojima *et al.*, 1995). This implies that any response due to direct electron transfer from lactate oxidase to polypyrrole would soon decrease since it is known from the horseradish peroxidase enzyme assay that hydrogen peroxide is produced by the LOD/PPy track-etch membrane in the presence of L-lactate and oxygen.

The enzyme entrapment either within the tubules or on the surface is reflected by the response to L-lactate. Overall, the highest enzyme response obtained from the horseradish peroxidase assay was from track-etch membranes with medium sized pores which could immobilise the lactate oxidase well and the highest flow injection response was observed from membranes with large pores. Koopal (1992) found that enyzme immobilisation was

most effective inside the tubules and not on the track-etch membrane surface. A tubule of the correct size may allow lactate oxidase to freely enter and then bind to the corrugated polypyrrole surface. It can be seen from the table that the enzyme activity of the track-etch membranes did not correlate to the FIA current response to lactate. For example the 800 nm, 30 s membrane has a low enzyme activity but a large current response when examined in the flow injection system. This could indicate that lactate oxidase was embedded deep in the track-etch membrane tubules and when in contact with L-lactate, responded in a way which could be seen electrochemically, but not by the enzyme assay. The lactate oxidase may use the polypyrrole as an electron acceptor instead of oxygen during the catalysis of lactate to pyruvate and direct electron exchange may occur. Direct communication between an oxidase enzyme (glucose oxidase) and polypyrrole was suggested by Koopal *et al.* (Koopal, 1992; Koopal *et al.*, 1992b). However, there is a distinct trend of decreasing electrochemical signal with increasing polymerisation time. This indicates that lactate oxidase in the presence of L-lactate is producing hydrogen peroxide which is diffusing through the tubules and being detected at the electrode surface. The polypyrrole restricts the diffusion thus more polypyrrole results in lower currents. The linearity of detection would also be increased by inhibiting the diffusion (see Chapter 4) and this trend is also observed. A good illustration is the 800 nm track-etch membrane. The ratio of response of 1 to 5 mM L-lactate increases from 2.5 to 5 with increasing polypyrrole thickness, thus showing that the linearity was increased. This is in agreement with Belanger *et al.* (Belanger *et al.*, 1989; Fortier *et al.*, 1990) and Kuwabata and Martin (1994) who found that polypyrrole acts as an inert immobilisation film for the enzyme glucose oxidase and limits the current response from glucose. Kuwabata and Martin (1994) found that the base electrode, sputtered platinum on carbon, gave higher responses to glucose than when polypyrrole and glucose oxidase were immobilised on the surface.

The response of the lactate oxidase modified track-etch membrane to L-lactate was very stable. This was illustrated by the small standard deviations of ten responses. This would indicate that the lactate oxidase is well protected within the polypyrrole matrix and is not being denatured by any hydrogen peroxide produced. The protective effect of polypyrrole has previously been observed with glucose oxidase which operated in the presence of

hydrogen peroxide for twice as long in the polypyrrole matrix as soluble or chemically-immobilised glucose oxidase (Fortier & Belanger, 1991).

The large variation in flow injection responses between different track-etch sections of the same membrane is probably due to an uneven polypyrrole coverage. Polymerisation took place where polypyrrole and iron (III) chloride solutions came into contact, but may not have occurred homogeneously over the track-etch membrane. The surface of the membranes were visually uneven; polypyrrole forms as a black layer and this was generally denser one side of the track-etch membrane than the other. This would lead to uneven lactate oxidase immobilisation across the surface of the track-etch membrane and thus the signal would vary.

There were no discernable peaks on the cyclic voltammogram of the track etch membrane, this would indicate that the polypyrrole was electrochemically inactive. Belanger and colleagues (Belanger *et al.*, 1989; Fortier *et al.*, 1990) and Kojima *et al.* (1995) have carried out cyclic voltammetry on polypyrrole and shown its deactivation by hydrogen peroxide or by increasing the potential above 0.55 V (SCE). It was also reported that polypyrrole becomes electro-inactive from nucleophilic attack by water or anions (Belanger *et al.*, 1989) and so the track-etch membranes in this study could have become inactive due to aging in buffer.

If FAD was in contact with polypyrrole, peaks should have been visible on a cyclic voltammogram with conducting polypyrrole. Flavine adenine dinucleotide, free in solution, is electrochemically active with a redox potential around -480 mV (Ag/AgCl, 1 M KCL) and FAD attached to a glassy carbon electrode was still active (half wave potential -440 mV (Ag/AgCl, 1 M KCl)) (Narasimhan & Wingard, 1986). No peaks were observed on the cyclic voltammogram from this study so it can be assumed that FAD was not free to communicate with the electrode. From this it is assumed that one of two situations occurred. Either FAD was closely bound to the enzyme and protected by the enzyme's outer shell, or FAD was in contact with polypyrrole, but because polypyrrole was not conducting, no redox activity was observed. Although Belanger *et al.* (1989) observed an extra redox couple at approximately -470 mV (SCE) when glucose oxidase was incorporated into polypyrrole, they allocated it to immobile irreversibly oxidised polypyrrole units since the addition of

FAD did not affect the cyclic voltammogram. Unfortunately, Kojima *et al.* (1995) did not describe the nature of the cyclic voltammograms they obtained.

Utilising the concept of lactate oxidase immobilised within a conducting polypyrrole matrix, screen-printed electrodes were fabricated. This involved electrochemically forming polypyrrole within a porous membrane of uniform latex particles held in place by an agarose gel on the surface of an electrode. Lactate oxidase was immobilised within the polypyrrole coated pores and then the matrix was ground to form a powder which could be incorporated into a printing ink. This is the first report of lactate oxidase used with polypyrrole to form a conducting printing ink. Although reports of this matrix with glucose oxidase have been successful (Koopal *et al.*, 1994; Koopal *et al.*, 1992a), the response to L-lactate upon immobilising lactate oxidase was poor. As outlined earlier (in Section 2.1.), the interaction between the active site of an enzyme and an unnatural mediator is quite specific. Lactate oxidase may not interact with polypyrrole in the same manner as glucose oxidase and this could explain the lack of response observed from the screen-printed electrodes. It is also possible that the amount of platinum was reduced and therefore the hydrogen peroxide detection was lowered.

2.4.6. Comparison of the Response to Hydrogen Peroxide, Lactate and Ascorbic Acid in Terms of Current Density.

When considering current densities of modified electrodes, the electrode's electrochemical area rather than the geometric area is significant in determining its catalytic efficiency. An increase in reactive surface-area not reflected by an increase in geometric area will increase the observed current signal, without being catalytic. Polypyrrole and PB modified electrodes were not included in this study since it had already been established that these chemistries were not viable for a stable and reproducible lactate sensing device.

The charge at very short times was distorted by the double layer charging process and after 1 second probably by natural convection due to large variations in concentration. These effects have been well documented (Bard & Faulkner, 1980). It can be seen form Table 2.6. that the reproducibility of the screen-printed electrodes is greater than the hand-fabricated electrodes. The same platinum disc electrode was tested ten times and gave a coefficient of

variation of 2.1%, suggesting that this was the error in measuring the electrochemical surface area by this method. The screen-printed electrodes gave coefficients of variation less than 6% for four different electrodes, demonstrating that the screen printing process can be used to reproducibly manufacture devices. The electrochemical surface area of all electrodes measured was larger than the geometric area. This is due to the surface topography, the electrodes have a rough surface and may be porous. The ratio of geometric to real surface area is known as the roughness factor and is usually greater than unity for solid electrodes (Plambeck, 1982). The roughness factor for the platinum disc electrode was found to be 1.108 and this is consistent with other reports (Loughran, 1994).

2.4.7. Conclusions.

2.4.7.1. General Conclusions.

A fast and simple method for forming a hexacyanoferrate film onto carbon was used to modify hand-fabricated and screen-printed electrodes. The hexacyanoferrate films improve electro-catalysis of hydrogen peroxide at carbon and reduce interference from ascorbic acid, although the effects were reduced over time. The immobilisation of lactate oxidase onto the surface of modified carbon hand-fabricated electrodes enabled L-lactate concentrations in buffer solutions to be measured quickly and easily, providing real-time information.

By combining Prussian Blue with a carbon ink, screen-printed electrodes could be easily fabricated. Although the PB ink was electro-catalytic towards hydrogen peroxide, the response was not as great as other carbon modifications, and the ink was not chemically stable, tending to loose activity over time. In conclusion, PB screen-printed electrodes appeared to offer no sensing advantages over the hexacyanoferrate film-modified electrodes. Further investigations into L-lactate detection by immobilising lactate oxidase on PB modified-carbon electrodes, were therefore discontinued.

Electrodes with a stable and reproducible response to hydrogen peroxide were fabricated with rhodinised carbon, employing the screen printing process. Interference from ascorbic acid was lower at rhodinised-carbon than at unmodified-carbon screen-printed electrodes, at the optimum potential for hydrogen peroxide detection. The electrodes were easily combined with lactate oxidase to sensitively detect L-lactate in buffer solutions.

Tetrathiafulvalene was used as an artificial mediator between lactate oxidase and carbon electrodes and succeeded in lowering the working potential of L-lactate detection. Although a successful attempt was made to screen printed electrodes employing tetrathiafulvalene and lactate oxidase, the response to L-lactate at +200 mV (SCE) was unstable and the interference from ascorbic acid was greater than that obtained at unmodified-carbon screen-printed electrodes.

A novel method for transducing the signal from lactate oxidase to the electrode was attempted in the form of polypyrrole. The immobilisation of lactate oxidase within a polypyrrole matrix provided a relatively stable environment which could be used in combination with the screen printing process to mass fabricate electrodes. From the results gained in this thesis, it is not possible to entirely eliminate the possibility of direct electron transfer from lactate oxidase to polypyrrole, but it can be assumed that if any does take place, it is in combination with hydrogen peroxide detection. The majority of the signal is due to hydrogen peroxide oxidation at the electrode surface. It can be concluded that further investigation into the mechanism of lactate detection is needed to clarify the sensor operation in order to improve its response.

2.4.7.2. Selection of Electrode Transducer Chemistry.

One transducer chemistry was to be chosen for further investigation. Four main factors influenced this decision:-

- cost
- ease of manufacture
- sensitivity
- selectivity

Manufacturing cost is an important consideration in the commercialisation of disposable sensors, especially for single-use devices used *in vivo* or *ex vivo* (Kost & Hague, 1996). The cost-effectiveness of screen printing has been recognised and is now in wide use(Alvarez-Icaza & Bilitewski, 1993; Bilitewski *et al.*, 1992; Cardosi & Birch, 1993; Cardosi & Turner, 1990; Hart *et al.*, 1996; Rohm *et al.*, 1996; Sprules *et al.*, 1995). The low cost of the initial sensor design and continued cost reduction over time due to bulk

buying and modest waste are favourable qualities. The raw materials, that is the components of the sensor, are the main constituents of the cost. Although carbon itself is inexpensive, the modifiers can be very expensive.

For easing manufacture and increasing reproducibility, mass fabrication technologies need to be used. The physical robustness and response stability of the transducer needs to be taken into consideration (Vadgama, 1992) both during fabrication and operation.

The sensitivity of a biosensor should be sufficiently high to allow convenient measurement of the transducer output signal with electronic instrumentation . If the sensitivity is high, the extension of the linear range can be achieved by the application of a diffusion limiting membrane.

Although carbon is used in many biosensor devices, the response to ascorbic acid is greater than the hydrogen peroxide response at most potentials used, hence restricting its use for *in vivo* applications. Simple modification of carbon electrode working surface reduces the working potential needed to measure L-lactate at enzyme electrodes. However, the modification of carbon may also lower the over-potential needed to oxidise ascorbate and therefore a compromise has to be sought between enhancing the signal to L-lactate and lowering the oxidation of ascorbate.

Potassium hexacyanoferrate and Prussian Blue were the cheapest chemistries used although both modifications of carbon produced unstable sensors during operation. Although tetrathiafulvalene modification gave a high sensitivity to L-lactate (upon lactate oxidase immobilisation), the interference from ascorbate was very high. Tetrathiafulvalene may also have limitations in sensing applications due to its toxicity and the possibility of leeching out of the sensor. Rhodinised carbon was the most expensive chemistry, but was the easiest material to handle and indeed was the most chemically and physically stable. Since it had a very high sensitivity to L-lactate (upon lactate oxidase immobilisation) and low sensitivity to ascorbate, a diffusion barrier could be applied to enhance the signal. Rhodinised carbon was selected as a promising transducer chemistry and further studies on this and other noble metal-modified carbons were carried out.

CHAPTER 3:

NOBLE METAL-MODIFIED CARBON ELECTRODES

3.1. INTRODUCTION.

The investigation into amperometric transducer chemistry, described in Chapter 2, suggested that biosensors based on rhodinised carbon had the most favourable characteristics. This chapter therefore assesses noble-metal modified-carbon electrodes for use in mass fabricated, disposable sensors for selective and sensitive detection of L-lactate. Other noble metal-modified carbons were explored in order to improve the response to hydrogen peroxide at the rhodinised-carbon electrodes evaluated in Chapter 2. Palladium, rhodium and platinum modified carbons were investigated for this purpose. The responses to hydrogen peroxide at various potentials were measured and interference from ascorbic acid was also addressed. This lead to rhodium on carbon to be chosen for the development of an L-lactate enzyme electrode.

The use of noble metals (platinum, rhodium, palladium, gold etc.) coated onto carbon to create catalytic materials has been widely adopted. Methods for incorporating the metal into the carbon matrix include:-

- cyclic voltammetry (Dong *et al.*, 1993; Gunasingham & Tan, 1989a; Hart *et al.*, 1996; White *et al.*, 1994b),
- galvanometry (Heider *et al.*, 1990),
- sputtering (Gorton, 1985; Gorton & Svensson, 1986; Jonsson & Gorton, 1987),
- evaporation (Johnston *et al.*, 1995),
- straightforward mixing of powdered metal into a carbon ink (Wang *et al.*, 1995c; Wang *et al.*, 1995d).

Metallised graphite powders are also commercially available and can be mixed with binders to form both modified carbon paste electrodes (Wang *et al.*, 1995a; Wang *et al.*, 1994; Wang *et al.*, 1992b) and thick-film electrodes (Collier *et al.*, 1996; Newman *et al.*, 1995; White *et al.*, 1996).

It is reported that dispersed metal microparticles exhibit improved electrocatalysis over pure metal surfaces (Dong & Qui, 1991; Gorton & Svensson, 1986; Shimazu *et al.*, 1987; Wang *et al.*, 1995b; Wang *et al.*, 1992b). There is electronic interaction and synergistic effects between the catalyst and the support, the metal acts as an electron donor to the support. For

example, platinum has been demonstrated to donate electron density to its carbon support (Mukerjee, 1990). Gorton and Svensson (1986) investigated the amperometric detection of hydrogen peroxide at sputtered palladium and gold on carbon electrodes. They found that the decrease in the overvoltages depended on both the layer thickness of the catalyst (palladium) and on the background material (graphite or glassy carbon). The particle size and structural character of the metal microparticles affects electrocatalysis: a highly catalytic surface is generated as long as the particle size of the deposited metal is comparable to the thickness of the electrical double layer (Mukerjee, 1990).

The oxidation of hydrogen peroxide at platinum electrodes was described by Lingane and Lingane (1963) as:-

$$Pt(OH)_2 + H_2O_2 \rightarrow Pt + 2H_2O + O_2$$

$$Pt + 2H_2O \rightarrow Pt(OH)_2 + 2e^- + 2H^+$$

with an overall reaction of $H_2O_2 \rightarrow 2H^+ + O_2 + 2e^-$.

Hydrogen peroxide reduces the metal oxide film to the metal which is re-oxidised electrochemically. Gorton (1985) carried out work on carbon rod electrodes sputtered with palladium and gold and amperometrically detected hydrogen peroxide at these surfaces. His experiments showed the oxidation of hydrogen peroxide to occur by the same pathway as that described by Lingane and Lingane. This suggests that the electrochemical oxidation of hydrogen peroxide proceeds in this manner on all platinum group metals, although it is not clear from experimental evidence whether or not this is a correct assumption (Wang *et al.*, 1994).

A number of lactate enzyme electrodes that utilise metal-modified carbons have been reported over the period of this work illustrating how attractive this approach is for the development of amperometric sensors.

3.1.1. Aims.

The following list highlights the aims of this chapter:-

- to investigate the current-potential response of a range of noble-metal modified-carbon electrodes (carbon modified with platinum, rhodium or palladium) with hydrogen peroxide;
- to study the response of the best metallised carbon to hydrogen peroxide and ascorbic acid at a range of potentials (to oxidise or reduce hydrogen peroxide);
- to immobilised lactate oxidase with the chosen metallised carbon and examine the sensing behaviour to L-lactate;
- to chose an optimum operating potential for this sensor.

3.2. EXPERIMENTAL.

3.2.1. General Reagents.

All general reagents used were as described in Section 2.2.1.

3.2.2. Metallised-Carbon Electrode Fabrication.

Rhodium and palladium on carbon (metallised carbon, 5% w/w metal on graphite) were purchased as powders from Aldrich Chemical Company (Gillingham, Dorset) and Avocado Research Chemicals (Heysham, Lancashire). The metallised carbon was crushed using a pestle and mortar to produce a finer powder and precautions were taken to avoid contact or inhalation of metallised carbons. The ink formulation described by White (1993) was used as a basis for this work. A screen-printable ink was made by dispersing the metallised carbon powder in a solution of hydroxyethyl cellulose (HEC, 2% w/v in buffer) with a small amount of finely milled carbon powder in a weight ratio of 1:1:3 (respectively) to make a smooth paste. The catalytic powders from MCA (Melbourn, Cambridgeshire) were mixed with HEC (2% w/v in buffer) in a weight ratio of 1:2. The inks were printed onto the eight array electrodes using the water-resistant working electrode screen with the DEK 245 screen printer as previously described (section 2.2.4.). The electrodes were dried at room temperature for 2 hours and stored in the dark. A cellulose acetate outer membrane was applied by dip coating the electrodes in a 2% (w/v in acetone) solution and allowing them to dry for 1 hour before testing commenced.

3.2.3. Electroplating Rhodium.

The method used was taken from White (1993). Rhodium atomic adsorption standard solution (1025 $\mu g.ml^{-1}$ rhodium in 5% w/v hydrogen chloride, from Aldrich Chemical Company, Gillingham, Dorset) was diluted in water to a concentration of 10.25 $g.ml^{-1}$ and potassium hydroxide added to raise the pH to 4.5. A three electrode system was used, a saturated Calomel reference electrode (SCE, Russell, Auchtermuchty, Fife), a platinum wire auxiliary electrode (0.5 mm, BDH Limited, Poole, Dorset) and a screen-printed carbon working electrode. Rhodium solution (20 ml) was placed into a beaker into which the three electrodes were immersed. Cyclic voltammetry was undertaken using the Autolab (EcoChemie, Utrecht, Netherlands) in the voltammetry mode of the GPES3 program. The start and end potential of

the scan was varied, from -1 to +0.5 V (SCE) with a scan rate of 0.05 $V.s^{-1}$; ten scans were carried out. Electrodes were then washed in water and stored dry, in the dark. The solution was changed and the procedure repeated for the next electrode.

3.2.4. Lactate Oxidase Enzyme Electrode Formation.

Lactate oxidase (LOD, E.C.1.1.3.2., ex. *Pediococcus*, 35 U.mg lyophilised powder from Genzyme Limited, Kent) and carbon powder (T15 graphite from Lonza G and T Limited, Sins, Switzerland) were dispersed in hydroxyethyl cellulose solution (HEC, 2% w/v in buffer) in a weight ratio 1:10:30 (LOD:T15: HEC). This ink was mixed on a rotational stirrer for 30 to 60 minutes before printing commenced using the water-resistant working-electrode screen as previously described (section 2.2.4.). The lactate oxidase electrodes were dried at room temperature for approximately 1 hr. A retaining membrane of cellulose acetate (2% w/v in acetone) was applied over the electrode surface by dipping the electrode into the cellulose acetate solution and allowing it to dry vertically for at least one hour.

3.2.5. Amperometric Calibrations.

Hydrogen peroxide, L-lactic acid and ascorbic acid calibrations were carried out in the following manner. A three electrode system comprising a working electrode, saturated Calomel reference (SCE, Russell, Auchtermuchty, Fife) and platinum wire auxiliary (0.5 mm, BDH Limited, Poole, Dorset) was used in conjunction with a computer controlled electrochemical analyser (Autolab, EcoChemie BV, Utrecht, The Netherlands) in amperometric mode (GPES3 software). The electrodes were immersed in a 10 ml stirred buffer solution and the operating potential applied (generally +400 mV (SCE)). Once a steady background current was achieved, a known volume of sample solution (buffer containing either 10 M hydrogen peroxide, 0.2 M lithium L-lactate or 0.5 M sodium salt of L-ascorbic acid) was injected into the cell, away from the working electrode. When a steady current was again reached, another aliquot of sample solution was added, resulting in a step-like response.

To remove oxygen from the solutions they were purged with pure nitrogen gas (British Oxygen Company) for 20 minutes and then a steady stream of bubbles was applied during testing. Similarly, pure oxygen (British Oxygen Company) was bubbled through solution for 10 minutes before, and then during the test, to obtain an oxygen saturated solution.

3.2.6. Measurement and Presentation of results.

This was carried out as previously discussed in Section 2.2.20. It was assumed that the hydrogen peroxide reactions obey Michaelis Menton-type kinetics on the electrode surface (Johnston *et al.*, 1995). A double reciprocal plot (i.e., equivalent to the Lineweaver-Burk plot) of current against hydrogen peroxide concentration was constructed. A straight line with a positive gradient and positive intercept was taken to represent the following equation:

$$\frac{1}{i_{ss}} = \frac{K_M'}{i_{max}} \cdot \frac{1}{C} + \frac{1}{i_{max}}$$

where i_{ss} is the steady state current response,

i_{max} is the maximum current obtainable when all sites are full,

K'_M is the apparent Michaelis Menton value (concentration of substrate which will elicit a current response half the value of the maximum current response),

C is the concentration of hydrogen peroxide in the bulk solution.

This lead to the determination of the maximum current and the apparent K_M of rhodinised and palladinised carbon and carbon graphite to hydrogen peroxide at +400 mV (SCE).

3.3. RESULTS.

3.3.1. The Amperometric Response Of Palladinised Crabon And Rhodinised Carbon To Hydrogen Peroxide.

The hydrodynamic voltammogram of palladinised carbon is shown in Figure 3.1. It can be seen that oxidation occurred above +200 mV (SCE) but the electro-catalytic effect was not very great. Reduction, seen occurring from +200 - 0 mV (SCE), was not very great either.

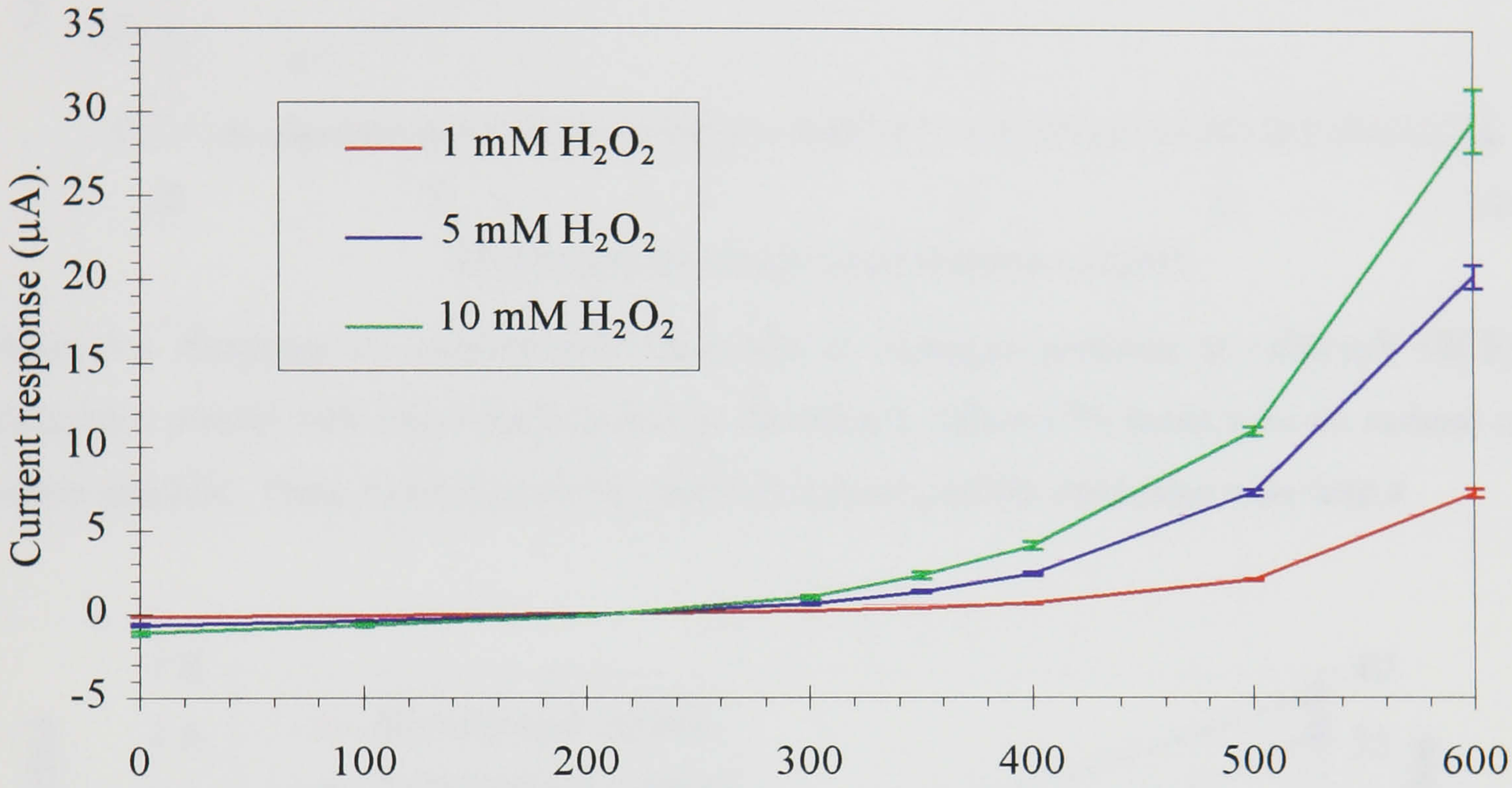


Figure 3.1. Hydrodynamic voltammograms of palladinised carbon screen-printed electrodes tested with hydrogen peroxide. Three electrodes tested.

When a calibration with hydrogen peroxide was carried out at +400 mV (SCE), the current response was much less than that observed at rhodinised carbon screen-printed electrodes (Figure 3.2.). The palladinised carbon response was only slightly improved over that of unmodified carbon. A double reciprocal plot of this data is shown in Figure 3.3. Good straight lines were obtained with regression parameters and the theoretical maximum obtainable current under saturating conditions and apparent K_M are listed in Table 3.1. The maximum currents were not achieved in practice at the modified carbon electrodes because the evolution of gas (oxygen) at the surface of the electrodes interfered with the signal. It can be seen that the maximum current and K_M were greatest for the rhodinised carbon although the palladinised carbon does show some improvement over the unmodified carbon.

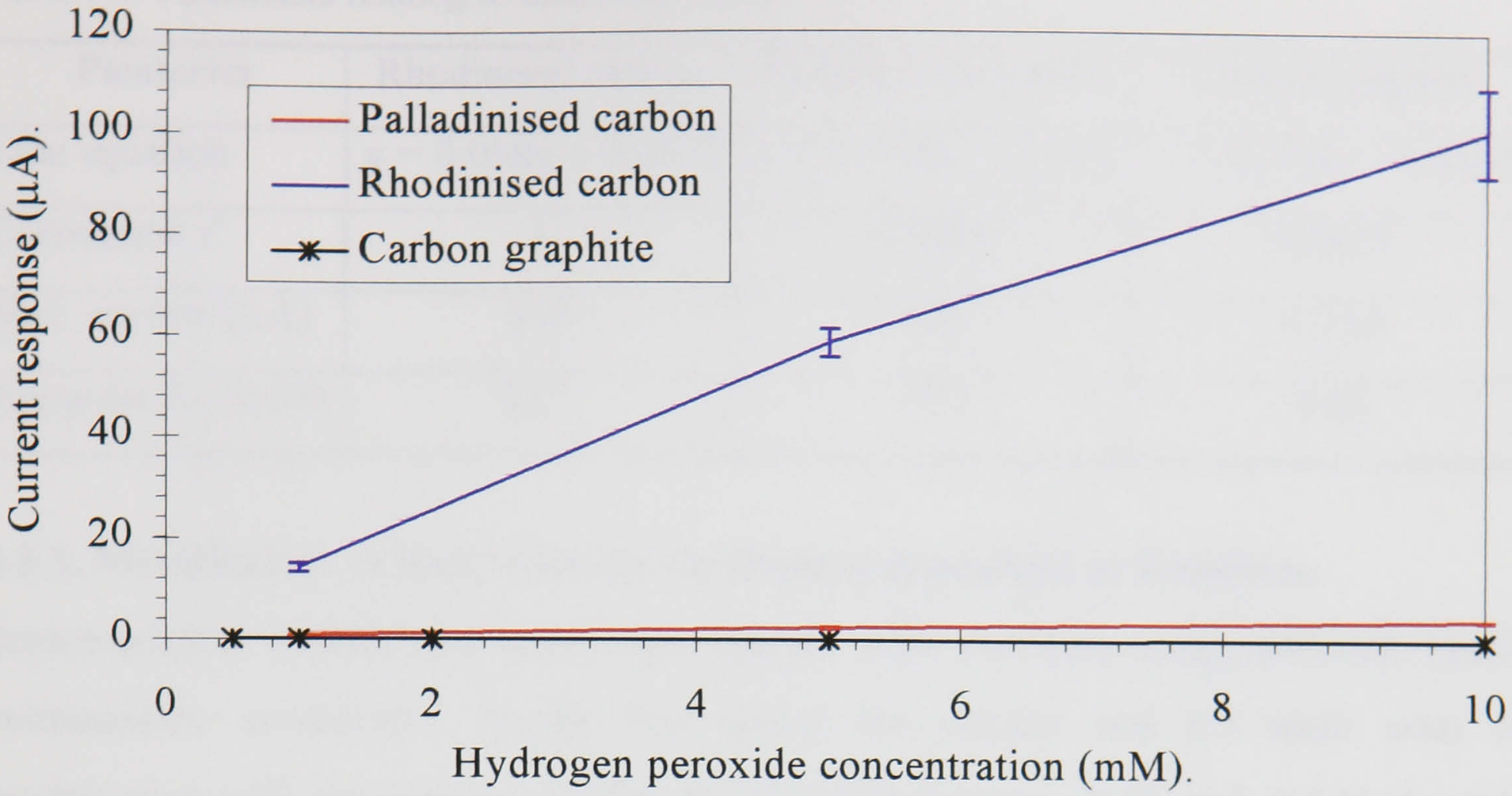


Figure 3.2. Response of screen-printed electrodes to hydrogen peroxide at +400 mV (SCE). Electrodes printed with either palladinised or rhodinised carbon (5% metal w/w on carbon) or carbon graphite. Three metallised carbon and two carbon graphite electrodes were tested.

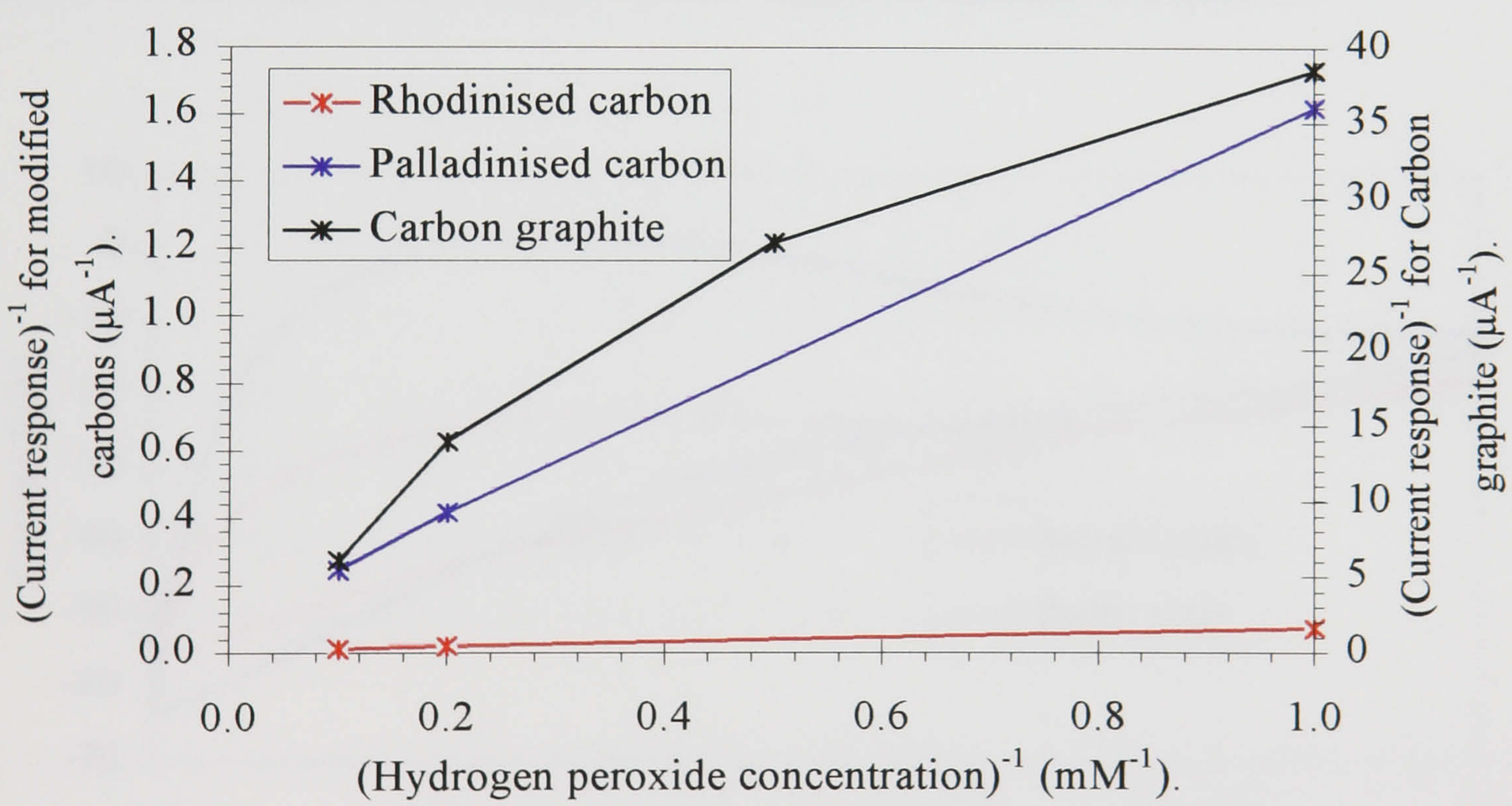


Figure 3.3. Double reciprocal plot of current response of metallised and unmodified carbon screen-printed electrodes to hydrogen peroxide at +400 mV (SCE). Data were taken from Figure 3.2.

Table 3.1. Parameters relating to data from Figure 3.3.

Parameter	Rhodinised carbon	Palladinised carbon	Carbon graphite
Line equation	$y = 0.068x + 0.0033$	$y = 1.5178x + 0.102$	$y = 32.752x + 6.4819$
Correlation r^2	1	0.9998	0.9875
Max. current (μA)	333	9.80	0.154
Apparent K_M (mM)	22.7	14.9	5.05

3.3.2. Modification of Electrodes by the Electro-deposition of Rhodium.

Screen-printed carbon electrodes were plated with rhodium using different cyclic voltammetric parameters. Figure 3.4. shows the second and the tenth scan of modification with rhodium by cycling the potential between -0.75 and -0.5 V at a rate of 0.05 $V.s^{-1}$. Hysterisis of the cyclic voltammogram occurred and much larger currents were observed. This trend was observed for all cyclic voltammogram procedures but lower currents were observed. Testing the modified carbon electrodes at +400 mV (SCE) with hydrogen peroxide gave results which are depicted in Figure 3.5.

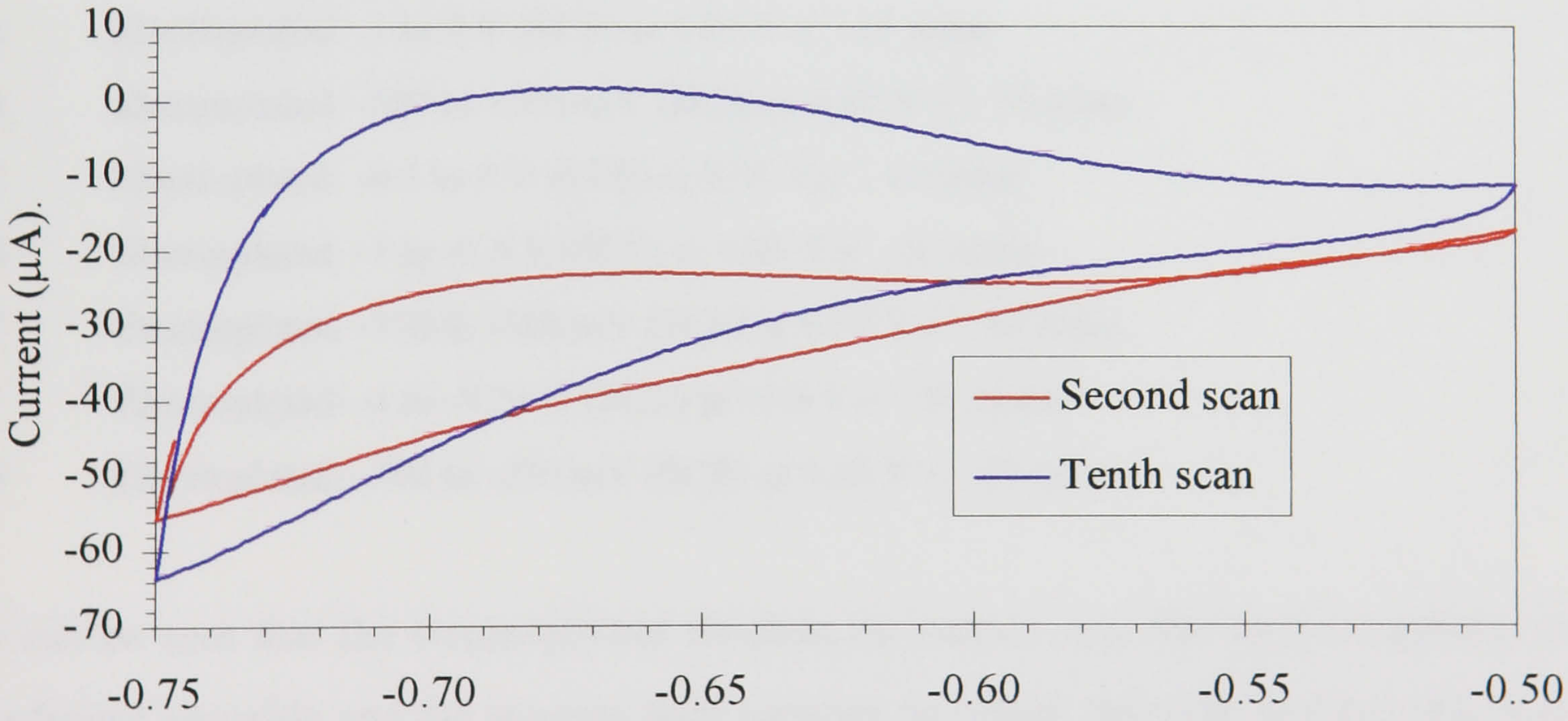


Figure 3.4. Typical cyclic voltammograms obtained from electroplating a carbon screen-printed electrode with rhodium. Procedure E (refer to Figure 3.5.), potential scanned from -0.75 to -0.5 V at a rate of 0.05 $V.s^{-1}$ in an aqueous solution of rhodium.

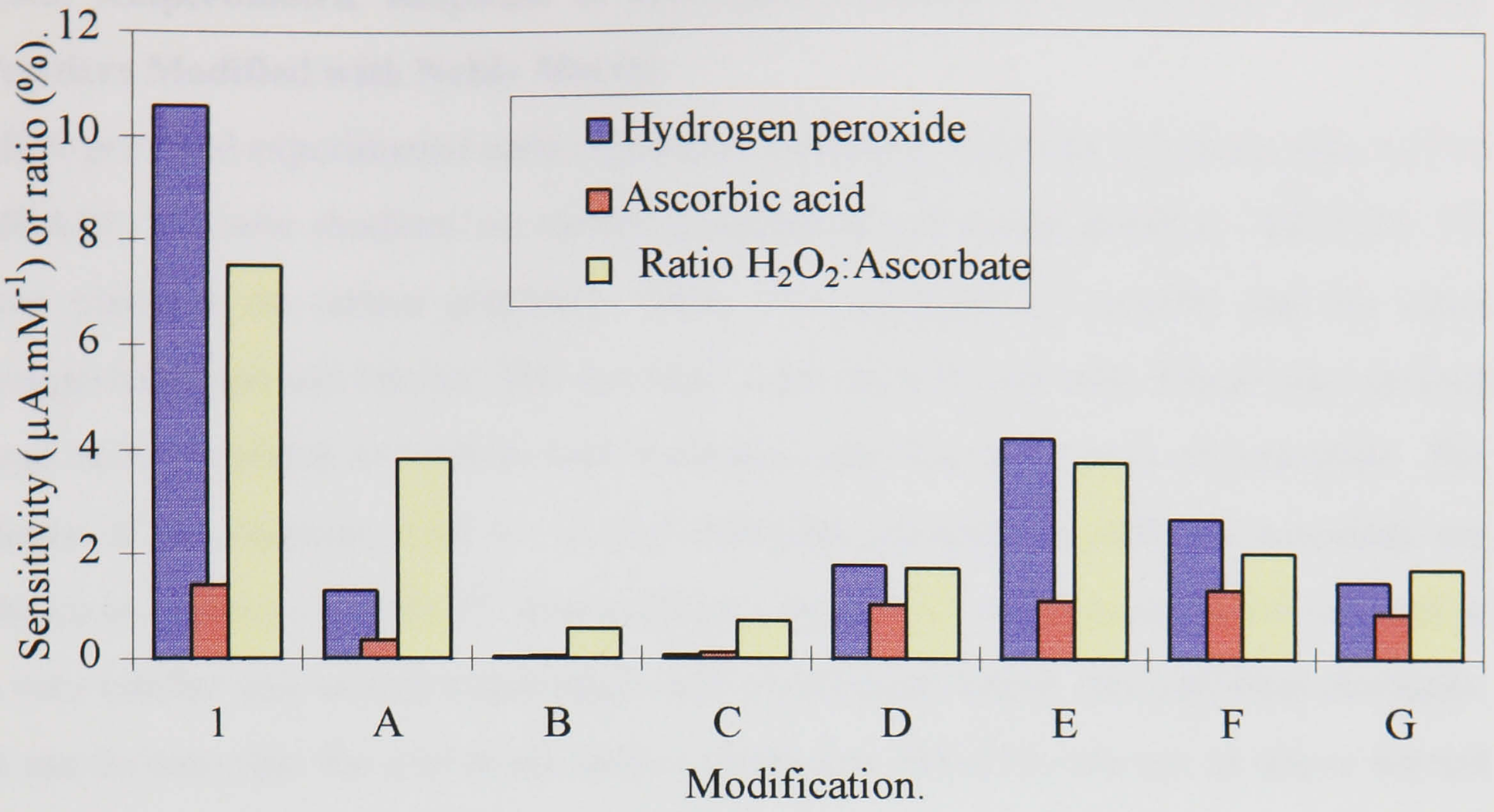


Figure 3.5. Response to hydrogen peroxide and ascorbic acid of modified screen-printed electrodes at +400 mV (SCE). Sensitivity calculated from the steady state current response to hydrogen peroxide (0-5 mM) in stirred buffer. Electrodes modified with rhodium on carbon in the following manner:

1	Screen-printed rhodinised-carbon ink (5% Rh on C)
A	Electroplated: -1 to 0 V (SCE) at 0.05 $V.s^{-1}$, 10 scans
B	Electroplated: -500 to +500 mV (SCE) at 0.05 $V.s^{-1}$, 10 scans
C	Electroplated: -0.5 to 0 V (SCE) at 0.05 $V.s^{-1}$, 10 scans
D	Electroplated: -1 to -0.5 V (SCE) at 0.05 $V.s^{-1}$, 10 scans
E	Electroplated: -750 to -500 mV (SCE) at 0.05 $V.s^{-1}$, 10 scans
F	Electroplated: -1 to -0.25 V (SCE) at 0.05 $V.s^{-1}$, 10 scans
G	Electroplated: -750 to -250 mV (SCE) at 0.05 $V.s^{-1}$, 10 scans

It can be seen that the screen-printed rhodium on carbon gave the largest response to hydrogen peroxide and the greatest ratio between hydrogen peroxide and ascorbic acid responses. Plating procedures A and E also gave high ratios, although the current responses were greater on electrodes modified by method E. It is interesting to note that although plating methods D, F and G gave a larger response to hydrogen peroxide than plating method A, the response to ascorbic acid was greater too, resulting in a lower ratio of hydrogen peroxide to ascorbic acid response.

3.3.3. Amperometric Response to Hydrogen Peroxide by a Number of Carbon Powders Modified with Noble Metals.

MCA provided experimental carbon powders containing promoted rhodium (MCA14 to MCA19, 5% w/w rhodium on carbon graphite) or promoted platinum (MCA20, 5% w/w platinum on carbon graphite). These were proprietary materials and the exact composition was not known. The powders were formed into inks which were printed onto base electrodes and tested with hydrogen peroxide at a range of potentials. The slopes of the response with 0 - 2 mM hydrogen peroxide at different potentials are shown in Figures 3.6. and 3.7. The electrodes made with rhodium on carbon respond in a very similar way to electrodes made with rhodinised carbon obtained from Avocado. It can be seen that the platinised carbon electrodes (MCA20) are not as active toward hydrogen peroxide as rhodinised carbon.

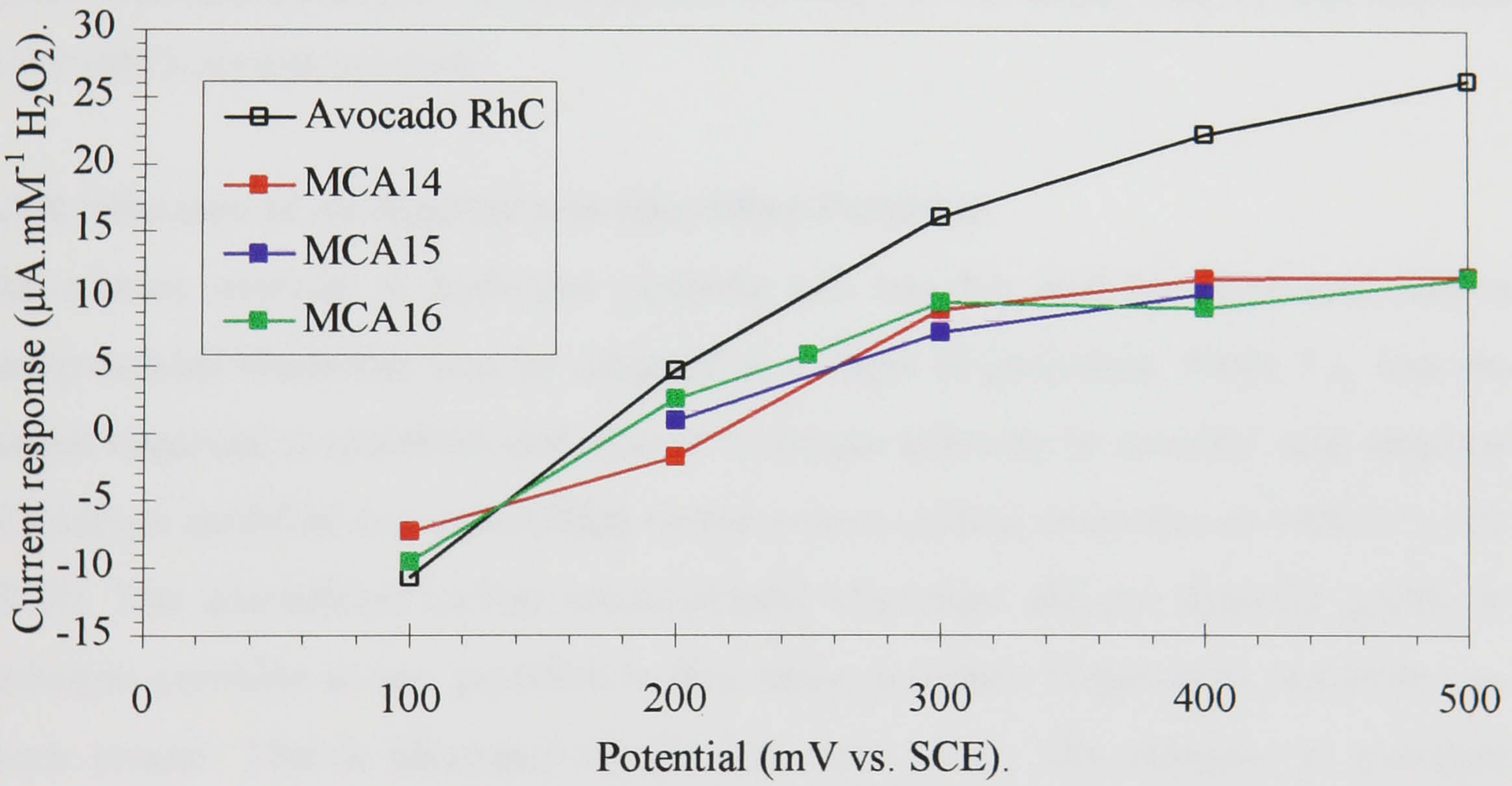


Figure 3.6. Hydrodynamic voltammograms of different screen-printing inks tested with hydrogen peroxide. Catalytic powders from MCA and Avocado formed into inks and screen printed onto base electrodes. Points represent the slope of the steady state current response to 0 - 2 mM hydrogen peroxide.

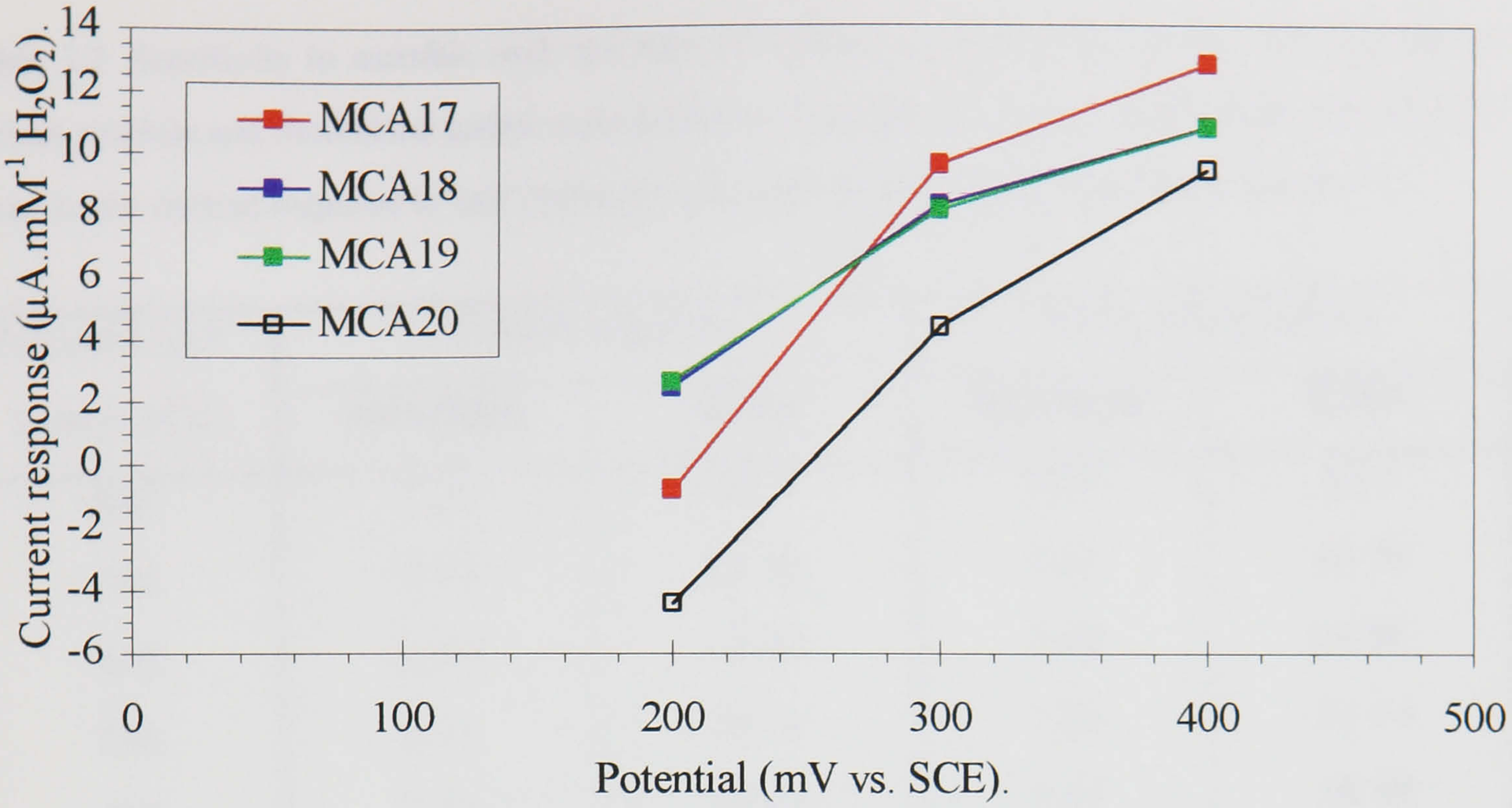


Figure 3.7. Hydrodynamic voltammograms of different screen-printing inks tested with hydrogen peroxide. Experimental catalytic powders from MCA formed into inks and screen printed onto base electrodes. Points represent the slope of the steady state current response to 0-2 mM hydrogen peroxide.

3.3.4. Selection of an Appropriate Operating Potential.

The current response to hydrogen peroxide and ascorbic acid by rhodinised carbon screen-printed electrodes was investigated at a range of potentials. Table 3.2. lists the current response to ascorbate and ratio of hydrogen peroxide to ascorbic acid response of rhodium modified and unmodified carbon screen-printed electrodes at +800 to 0 mV (SCE). The unmodified carbon screen-printed electrodes did not respond greatly to hydrogen peroxide at any potential in this range and their response to ascorbate was much greater. This is illustrated by the low ratio value. The response to hydrogen peroxide by the rhodinised-carbon screen-printed electrodes was enhanced greatly, as shown before, and it is interesting to see that at high potentials (greater than +300 mV (SCE)) the response to ascorbic acid was less at the modified than the unmodified electrodes. However, at lower potentials (+300 mV (SCE) and below) the ascorbic acid response was greater at the rhodinised-carbon electrodes than the unmodified carbon electrodes.

Table 3.2. Sensitivity to ascorbic acid and ratio of hydrogen peroxide to ascorbic acid response of carbon graphite and rhodinised carbon screen-printed electrodes at a range of potentials. Sensitivity is given as the current response to unit hydrogen peroxide/ascorbic acid concentration ($\mu A.mM^{-1}$).

Potential (mV versus SCE)	Carbon graphite		Rhodinised carbon	
	Ascorbate	**Ratio**	**Ascorbate**	**Ratio**
800	3.25	45.5	3.15	44.1
700	3.17	44.38	2.91	40.74
600	2.68	37.52	2.49	34.86
500	2.51	35.14	2.26	31.64
400	2.19	30.66	2.07	28.98
300	1.45	20.3	1.87	26.18
200	0.89	12.46	1.70	23.8
100	0.89	12.46	1.44	20.16
0	0.34	4.76	1.25	17.5

Two operating potentials in the low and high regions where the response to hydrogen peroxide levelled off, were investigated further. The oxidation of hydrogen peroxide at +400 mV (SCE) is shown in Figure 3.2. A potential of -100 mV (SCE) was also chosen. Hydrogen peroxide was reduced at this potential, giving a negative signal. Figure 3.8. shows the response of rhodinised-carbon screen-printed electrodes to hydrogen peroxide under N_2, O_2 and air atmospheres at -100 mV (SCE). It can be seen that although the background currents were different, the response to hydrogen peroxide was not affected.

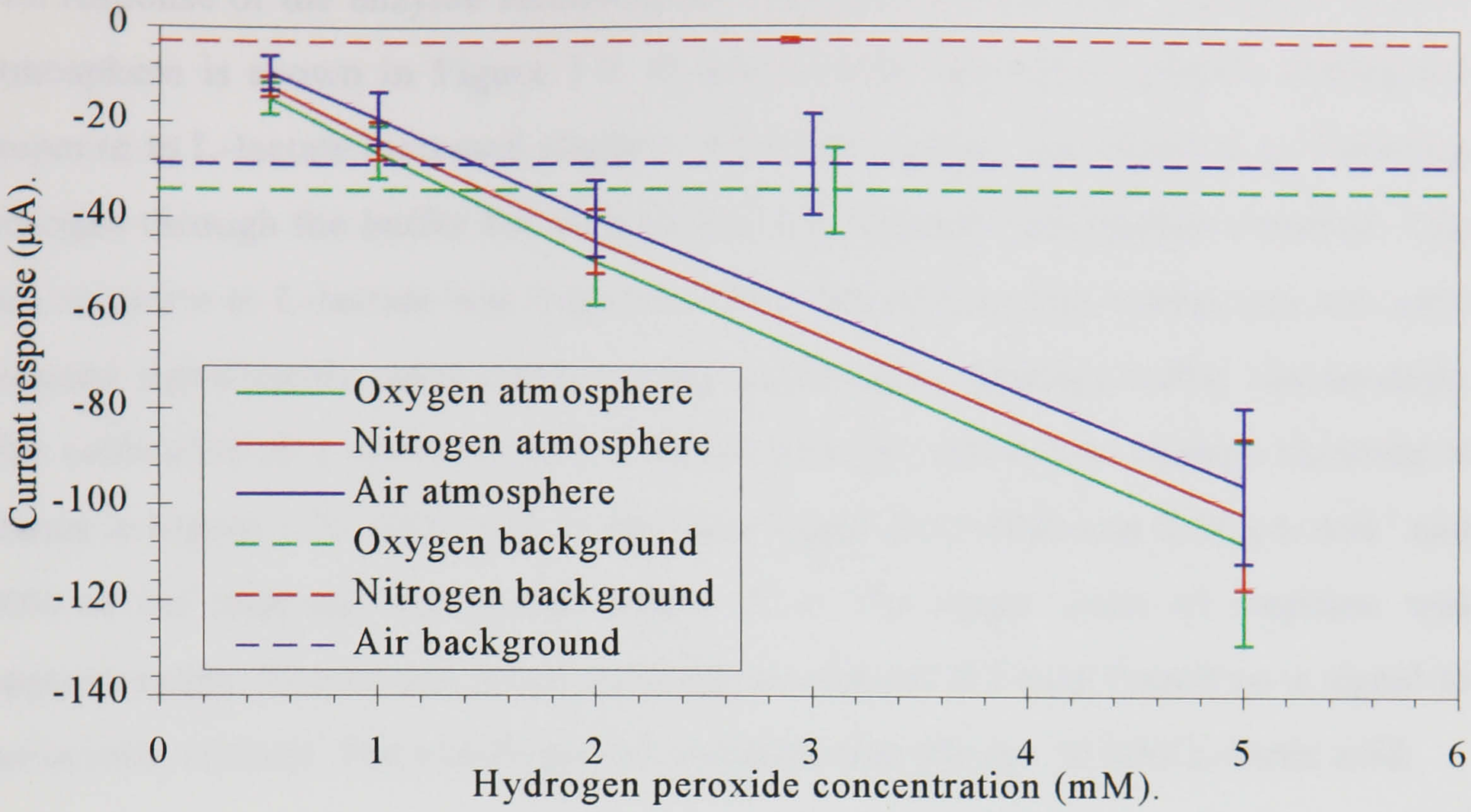


Figure 3.8. Current response of rhodinised carbon screen-printed electrodes to hydrogen peroxide at -100 mV (SCE). Error bars represent the standard error of the mean of 5, 6 and 7 steady state electrode responses in oxygen, nitrogen and air atmospheres respectively. Dashed lines represent the background currents of the electrodes in the respective atmospheres, with standard errors.

Screen-printed rhodinised-carbon and lactate oxidase electrodes were amperometrically tested with L-lactate at +400 mV (SCE) and produced large, positive currents in accordance with the oxidation of hydrogen peroxide produced by the enzymatic oxidation of lactate. When the enzyme electrodes were tested at +100 mV (SCE), a negative current was observed, in accordance with the reduction of hydrogen peroxide. However, when these enzyme electrodes were tested at 0 mV (SCE), the current response was positive. The response was not as great as that gained at +400 mV (SCE), nor was it linear, and it did not increase on applying potentials lower than -100 mV (SCE) (up to -400 mV (SCE) was tested and a very small positive current was observed on the addition of L-lactate). The response was repeatable but varied greatly between electrodes. The full response to L-lactate was regained when re-tested at +400 mV (SCE). When hydrogen peroxide was tested alone, it was reduced at all potentials below +190 mV (SCE) by these enzyme electrodes. The response of screen-printed rhodinised carbon electrodes to L-lactate was positive at these potentials but the magnitude was much less than that of the enzyme electrodes (nano amperes instead of micro amperes).

The response of the enzyme electrodes at +400 mV (SCE) under a reduced oxygen atmosphere is shown in Figure 3.9. It can be seen that upon repeated testing the response to L-lactate increased slightly. When the oxygen was depleted by bubbling nitrogen through the buffer for 20 minutes, the response was slightly lowered. The full response to L-lactate was regained when tested in aerated buffer and was only reduced significantly upon repeat testing in nitrogen saturated buffer (de-aerated). The calibration of a screen-printed rhodinised-carbon and lactate oxidase electrode is shown in Figure 3.10. The slope of the linear range (0-10 mM) was 0.21 $\mu A.mM^{-1}$ and 90% of the response was gained within 30 s. The upper limit of response was approximately 30 mM and lower limit approximately 0.5 mM (based on a signal to noise ratio of two). The coefficient of variation was 5% for 10 mM L-lactic acid.

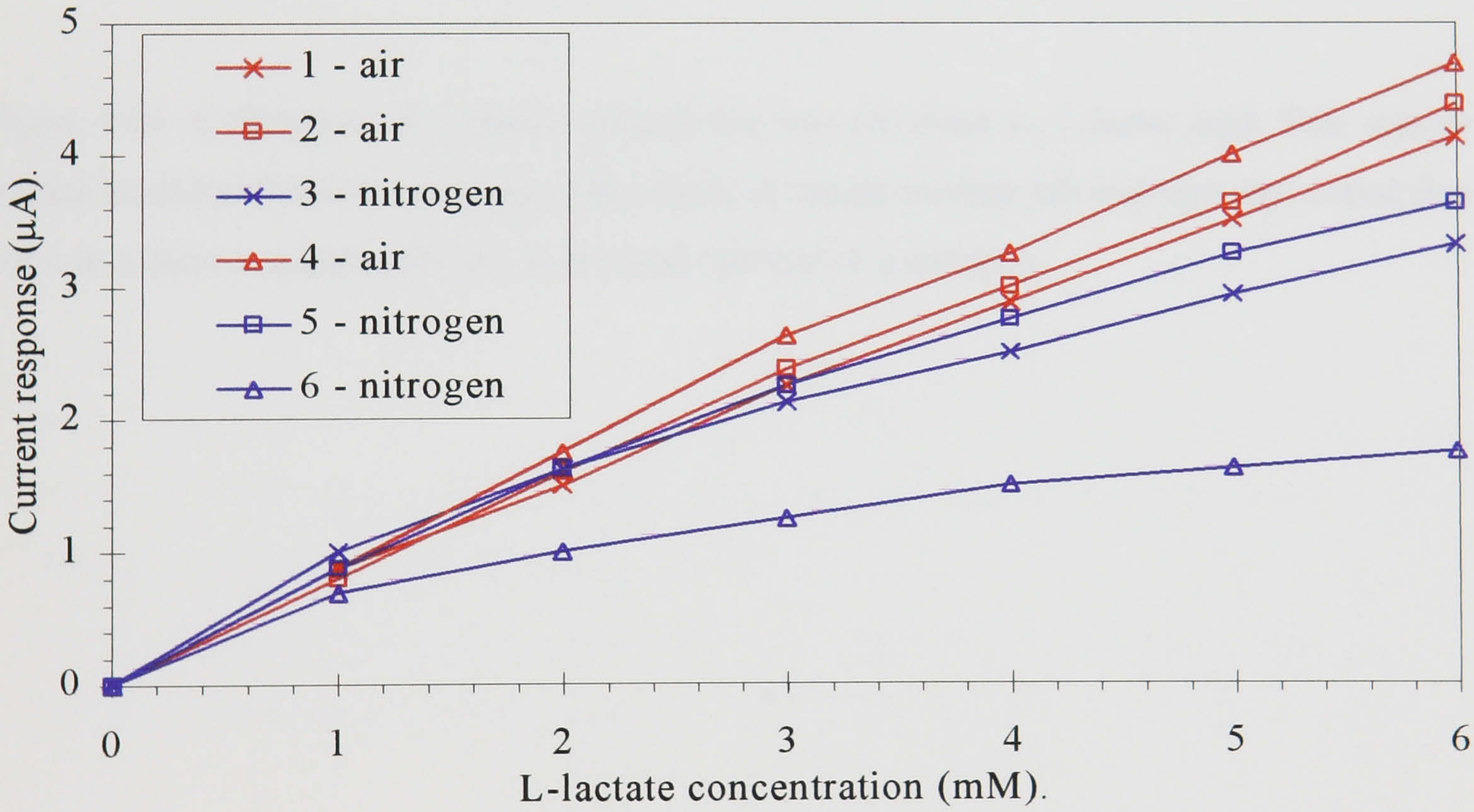


Figure 3.9. Response of a screen-printed lactate oxidase enzyme electrode under air or nitrogen atmosphere at +400 mV (SCE). Rhodinised carbon screen-printed with a layer of lactate oxidase ink and then dip coated twice in cellulose acetate (2% w/v in acetone).

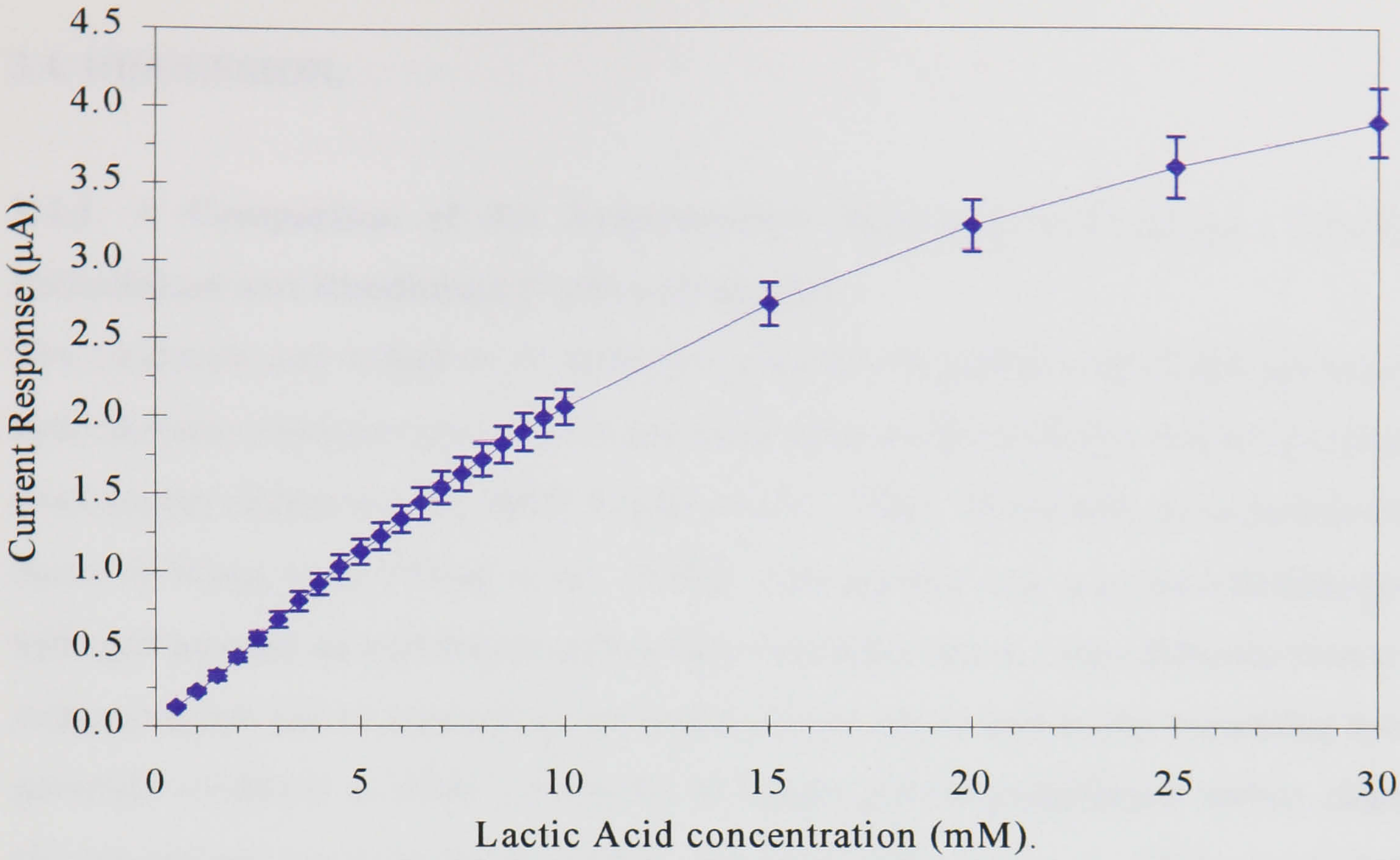


Figure 3.10. Calibration of a lactate oxidase enzyme electrode to L-lactic acid. One screen-printed rhodinised-carbon electrode with a layer of lactate oxidase ink and then dip coated five times in cellulose acetate (2% w/v in acetone) and tested three times.

3.4. DISCUSSION.

3.4.1. A Comparison of the Amperometric Response to Hydrogen Peroxide At Palladinised and Rhodinised Carbon Electrodes.

The oxidation and reduction of hydrogen peroxide on palladinised carbon shown by the hydrodynamic voltammogram follow the same trend as other reports, but the currents were much smaller (Johnston *et al.*, 1995; Sakslund *et al.*, 1996). The results are in agreement with those of Wang *et al.* (Wang *et al.*, 1992b), who showed that a cyclic voltammogram of hydrogen peroxide on palladinised carbon paste electrodes was not very different from a cyclic voltammogram ran in supporting electrolyte. When amperometrically measuring hydrogen peroxide oxidation at +400 mV (SCE) on screen printed palladinised carbon electrodes, electrocatalysis was only just noticeable. Johnston (Johnston *et al.*, 1995) report that non-activated palladium and gold electrodes at +400 mV (SCE) did not respond to peroxide whereas an activated electrode gave a response of 4 μA.mM^{-1} hydrogen peroxide at this potential. The application of a high potential activated the catalytic layer of palladium and gold electrodes and the activation seem to then be constant (Gorton, 1985; Johnston *et al.*, 1995). Activated electrodes showed a large increase in oxidation current above +200 mV (SCE) approaching a plateau at +400 mV (SCE) until approximately +700 mV (SCE) after which the response increased again (Gorton, 1985; Johnston *et al.*, 1995). This trend is also observed on palladium coated carbon (Sakslund *et al.*, 1996), although there was no mention of activation here. The aspect of activating palladium was not investigated in this thesis because it would ultimately involve an additional fabrication step which could be avoided by using rhodinised carbon.

The lowering of overpotentials was greater at the rhodinised-carbon than at the palladinised-carbon screen-printed electrodes; also the currents were larger. Oxidation of hydrogen peroxide started to occur at +200 mV (SCE) on palladinised carbon but at +190 mV (SCE) on rhodinised carbon. Calibration results can be analysed on the basis of Michaelis Menton-type kinetics (Johnston *et al.*, 1995). This is assuming that hydrogen peroxide adsorbs to the metal oxide sites according to a Langmuir isotherm which then undergoes electron transfer with the formation of a reduced metal site and release of the oxidation products. The electrochemical regeneration of the active site is assumed to be a non-rate-limiting step. The

results allow the comparison of kinetic parameters of the rhodinised, palladinised and graphite carbon electrode thereby quantitatively analysing each option. A potential of +400 mV (SCE) was chosen as the operating potential because the hydrogen peroxide response was just beginning to level off at rhodinised carbon and was just beginning to be noticeable at palladinised carbon electrodes. The results show that not only does rhodinised carbon have the largest apparent K_M and maximum current, but the correlation coefficient of one shows how precise the response toward hydrogen peroxide is. Johnston *et al.* (1995) reported on the kinetic parameters of palladium and gold electrodes with hydrogen peroxide. They show that, after activation, the electrodes had a K_M of 10.65 mM and a maximum current of 30 μA with hydrogen peroxide. Although the maximum current was higher than the values achieved with palladinised carbon in this work, both the K_M and maximum current were still lower than the rhodinised carbon values obtained here.

3.4.2. The Electro-catalytic Response of Electroplated and Commercially Produced Rhodinised Carbons to Hydrogen Peroxide.

This is the first report to compare electroplating rhodium onto carbon with screen printing prepared rhodinised carbon. The experiment illustrated that different methods of rhodinised carbon preparation gave different sensitivity to analytes. Wang *et al.* (1995b) also suggested this and hypothesised that not only will technique and potential affect the electocatalytic activity but duration, mass transport, media, pH and surface condition will also be relevant parameters to examine. Current responses to hydrogen peroxide were smaller at plated than printed electrodes. This is due to the rhodium content, ten scans was not sufficient to apply a thick enough catalytic layer to the carbon electrodes. White (1993) found that 70 scans gave the highest currents, although the plating potentials were slightly different (+100 to -900 mV (SCE)). Ascorbic acid was also tested, to observe how rhodium affected the response of carbon to interferences. The screen-printed electrode was coated with cellulose acetate which may have restricted the transport of ascorbic acid to the electrode surface, resulting in a lower response in its absence. The ratio of hydrogen peroxide to ascorbate sensitivity was used to normalise the responses so that a comparison could be made. The response to ascorbate did not follow the same trend as the hydrogen peroxide response showing that there is a complex relationship between the rhodium structure, content and its electrocatalytic effect. This relationship has been reviewed before (Mukerjee, 1990) although only limited information was

available; small particles possess structures very different from those at macroscopic levels and their influence required more detailed examination of the catalytic-support interactions of different metal particle sizes and shapes.

Screen printing rhodinised carbon involves a consecutive series of steps whereas electroplating involves a separate step which breaks up the production of sensors. The responses of the screen-printed electrodes were also better than the electroplated electrodes and therefore the screen printing of catalytic inks was taken further. Carbon powders, amenable to printing ink formation, containing promoted rhodium or platinum were a kind gift of MCA, Melbourn, Cambridgeshire. Rhodium was the metal of interest and platinum was used as a comparison. The catalytic powders showed very similar response trends and current densities to hydrogen peroxide. Platinised carbon gave the lowest sensitivity to hydrogen peroxide which did not level off at low potentials thereby justifying the use of rhodinised carbon. White *et al.* (1994b) also found rhodinised carbon to be superior to platinised electrodes in several respects, including selectivity and higher sensitivity for hydrogen peroxide. The Avocado rhodinised carbon showed the greatest current density to hydrogen peroxide, although the response did not level off as dramatically as the other rhodinised carbons. Data published by Newman *et al.* (Newman *et al.*, 1995) revealed that an MCA powder (MCA4) containing 5% rhodium had the best response compared to other metal-containing carbon powders (like platinum, ruthenium, palladium at various concentrations).

3.4.3. The Selection of An Operating Potential.

To further investigate the selectivity of rhodinised carbon, ascorbic acid responses were compared to hydrogen peroxide responses at a range of potentials on both the modified and unmodified carbon electrodes. This would also allow the judicious selection of an operating potential in a region of high hydrogen peroxide to ascorbic acid response ratio. Ascorbic acid was used because not only is it commonly found in biological media, but it evokes large currents which interfere with the analyte signal. The results showed that, not only did rhodinised carbon improve the response and produce a significant decrease in the overvoltage for hydrogen peroxide, but the ascorbic acid response was lowered at potentials above +300 mV (SCE) over unmodified carbon. The oxidation of ascorbic acid was higher at the rhodinised carbon than unmodified carbon at potentials below this, indicating a small shift in the oxidation potential to

lower values. This is confirmed by reports from Wang's group (Wang & Angnes, 1992; Wang *et al.*, 1994; Wang & Wu, 1995), who showed by cyclic voltammetry that the overvoltages were lowered at rhodium-coated surfaces with oxidation of ascorbic acid starting at +0.05 V (Ag/AgCl) (about 40 mV potential shift).

The selection of operating potential was based on a number of factors:-

- high response to hydrogen peroxide;
- low response to ascorbic acid;
- stable response to hydrogen peroxide if the potential changes slightly;
- other species (e.g., oxygen) would not interfere.

For these reasons, -100 mV (SCE) was chosen as an operating potential for the lactate sensor. However, when testing L-lactate in the presence of lactate oxidase, a very small positive current resulted. This current, apparently due to the oxidation of a species, resulted from the decrease in oxygen reduction current (associated with the enzymic consumption during L-lactate conversion to pyruvate) and was not due to an actual oxidation. The positive current is the net of the hydrogen peroxide production and oxygen depletion currents. Wang *et al.* report similar results at low potentials when testing lactate at rhodium dispersed carbon paste electrodes incorporating lactate oxidase (Wang *et al.*, 1994). When examining the base rhodinised carbon with hydrogen peroxide under different atmospheres, it was found that although the response did not change, the baseline currents were non-reproducible in both air and oxygenated solutions. This problem was effectively reduced by de-aeration with nitrogen, confirming the influence of oxygen on the response of the rhodinised carbon at this potential. This problem has also been recently reported by Sakslund *et al.* (1996).

Oxygen is required for the oxidation of lactate to pyruvate and since it interferes with the signal at -100 mV (SCE), this operating potential was excluded from further study. This led to the question of how much would a decrease in oxygen availability affect the operation of the sensor at +400 mV (SCE), the alternative potential to -100 mV (SCE). A study of the effect of de-aerating the solutions on the response of an enzyme electrode to L-lactate was undertaken. This showed that there was little change in the response, probably due to the entrapment of molecular oxygen within the sensor matrix. Only after considerable purging with nitrogen and

on a repeat test was the response lowered. It is unlikely that the sensor would be under such low oxygen concentrations for such a long period of time during operation.

It is interesting to note that the response to lactate in air actually increased upon repeat testing. This is attributed to the alterations in the structure of both the cellulose acetate membrane and the hydroxyethyl cellulose enzyme layer. Eventually the membranes would swell and become very porous, leading to the depletion of the enzyme (White, 1993). A lactate sensor dip coated five times in cellulose acetate gave a very reproducible signal at +400 mV (SCE) with a large linear range to L-lactate and high sensitivity. The application and effects of various membranes to limit the diffusion of analytes and interfering species was examined and the results are reported in the next chapter. A biocompatible and anti-fouling membrane needs to be applied for the operation of an implantable device and this was also investigated.

3.4.4. Conclusions.

This chapter has shown that rhodinised carbon is sensitive to hydrogen peroxide over a wide range, allowing an appropriate operating potential to be chosen. The rhodinised carbon response to ascorbic acid has been shown to be attenuated at potentials above +300 mV (SCE). An investigation into the application of membranes as a means of lowering the interference further was carried out in the following chapter (Chapter 4). In combination with lactate oxidase, membrane-coated rhodinised-carbon electrodes sensitively detected L-lactate over the clinically useful range. The useful employment of membranes to extend the linear detection range is also evaluated in Chapter 4. Screen printing has simplified the manufacture of modified carbon electrodes and experiments to optimise both the rhodinised carbon and enzyme ink formulations are addressed in Chapter 5.

CHAPTER 4:

MEMBRANE CHARACTERISATION.

4.1. INTRODUCTION.

Chapter 3 concluded that the application and use of membranes on the planar enzyme electrodes should be used to enhance their performance. This chapter discusses the ways in which the application of membranes can improve the sensor response in terms of sensitivity and selectivity. Furthermore a strategy was developed which could limit interference and provide some anti-fouling capability.

4.1.1. The Function of Membranes.

The three main functions of membranes identified for utilisation in this work were:-

- to improve sensor performance by increasing the linear range;
- to improve the selectivity of the sensor by excluding certain interfering compounds;
- to provide protection against protein adsorption.

Membranes have also acted as an enzyme immobilization matrix, to prevent flow dynamics from interfering with the signal and to add biocompatible properties to implantable needle sensors (Heineman, 1993; Mullen *et al.*, 1986; Shichiri *et al.*, 1987).

In 1978 Scheller *et al.* described the possibility of extending the measuring range of an enzyme electrodes by adding a substrate diffusion barrier. Pfeiffer *et al.* (1992) later showed a dependence of the sensor sensitivity on the barrier membrane thickness; a thicker membrane increased the linear range of detection but the sensitivity was reduced. The net result is a conversion from a kinetic controlled enzyme electrode to one which is diffusion controlled. Coarse porous membranes (pore size greater than 5 nm) are considered to have a sieve-like action with particles passing through by either a convective or diffusion mechanism. They comprise of either a well defined discrete cylindrical pore structure, for example polycarbonate and polyester membranes which have been perforated by nuclear track etching (Cyclopore and Nucleopore), or a less defined, sponge-like structure with a broad pore size distribution, for example, cellulose acetate and polyurethane (Tang & Vadgama, 1990).

Blood contains various readily oxidisable species which cause an interfering signal thereby posing a major problem for monitoring of L-lactate via electrochemical measurement of

hydrogen peroxide *in vitro*, *in vivo* or *ex vivo* (Lerner *et al.*, 1982; Vaidya & Wildins, 1994). To improve the selectivity of a sensor, interfering compounds can be excluded by means of a size exclusion and/or a charged membrane (Tang & Vadgama, 1990). A size exclusion membrane, formed with a low molecular-weight cut-off, is capable of rejecting many diffusible organic compounds and can indeed be manipulated to screen electrodes from small interfering molecules like ascorbic acid. Electrostatic membranes limit interference from charged species due to natural repulsion. Effective discrimination against ascorbic acid and other anionic interferents has been reported with perfluorinated and polyester ionomers (Nafion and Kodak AQ, respectively) (Wang & Golden, 1989; Wang *et al.*, 1984). Similarly, cationic coatings like poly(vinylpyridine), screen the underlying material from positively charged interferents like dopamine (Wang *et al.*, 1987). Significant improvement of selectivity can be achieved by using a combination of size and charge exclusion membranes (Vaidya & Wildins, 1994; Wang & Tuzhi, 1986).

The initial event after contact of a foreign material with blood is the deposition of a layer of protein at the blood-material interface (Lelah & Cooper, 1986); an interaction which may be irreversible (Elbert & Hubbell, 1996). Proteins shield their hydrophobic amino side chains from water by folding them towards the internal regions of the protein. If a foreign material has a hydrophobic surface, the protein may unfold, allowing the hydrophobic side chains to contact the surface thereby leading to essentially irreversible adsorption. Albumin is transported to the biomedical surface faster than any other plasma protein, consequently foreign surfaces are dominated by albumin (Elbert & Hubbell, 1996). In the case of irreversible protein adsorption, the first protein to arrive will cover the surface and so, in blood plasma, albumin will coat the foreign material. This fouling of the foreign material's surface creates another diffusion layer on a sensor, restricting the transport of the analyte (Abdel-Hamid *et al.*, 1995; Vaidya & Wildins, 1994), as well as starting a bioresponse cascade by the body (Lelah & Cooper, 1986). A study of protein adsorption is therefore an important prerequisite to an understanding of blood-material interactions, blood compatibility and the design and use of materials in invasive devices. If protection against protein adsorption is provided, then fouling of the sensor will be regulated.

4.1.2. Choice of Membranes.

The following polymers were considered for use as membranes in the construction of planar sensors for the determination of blood L-lactate.

Cellulose acetate was evaluated in this chapter for exclusion of interferents, ability to extend the linear range of detection and tested for protein adsorption. Cellulose acetate is a versatile membrane with size exclusion properties. It has been widely used in the fabrication of enzyme electrodes:-

- it has acted as an enzyme immobilisation matrix for glucose oxidase (Poitout *et al.*, 1991; Shichiri *et al.*, 1982);
- it has been applied to screen out interferents like ascorbic acid and uric acid (Anzai *et al.*, 1992; Bindra *et al.*, 1991; Mullen *et al.*, 1986; Wang & Hutchins, 1985);
- it has been used as a protective outer coating (Abdel-Hamid *et al.*, 1995; Maines *et al.*, 1996; Sittampalam & Wilson, 1983).

This is in addition to the diffusion restriction that the application of a membrane confers.

Nafion was employed in this chapter to exclude charged oxidisable species. It is a perfluorinated ionomer, produced by Du Pont and offers tremendous ion-exchange affinity for organic cations (Szentirmay & Martin, 1984). This perfluorosulphonic ion-exchange membrane material provides a negatively charged surface and thereby electrostatically repels negatively charged species like ascorbic acid and uric acid (Wang & Tuzhi, 1986). It has been commonly employed in sensor construction to exclude these oxidisable interferents (Heineman *et al.*, 1991; Newman *et al.*, 1995; Pan & Arnold, 1996; Wang & Tuzhi, 1986).

Pellethane, a polyurethane, was used in this chapter to encapsulate an L-lactate enzyme electrode and evaluate the lactate response in protein solutions, to judge the anti-fouling ability of the polymer. Thermoplastic polyurethane elastomers are a large group of biomedical polyurethanes. Early polyurethanes possessed poor hydrolytic stability, probably due to polyester polyols. Therefore incorporation of hydrolytically stable polyether soft segments was carried out. Pellethane is a polyether urethane containing aromatic isocyanate, which is essentially a rigid molecule, and results in a segmented elastomer. It is a thermoplastic, developed by Up-john Co. (New Haven, Connecticut) which can be cast from

solvents (Lelah & Cooper, 1986), but it is no longer available for implant applications, due to the lack of long term data on the performance of polyurethanes and the liability associated with implantation (Pinchuk, 1994). Due to their excellent physical and mechanical properties, and relatively good blood compatibility, polyurethanes have been favoured for biomedical applications. Although polyurethanes do not show as good biocompatibility as other materials (like poly(HEMA)), there are still no better elastomeric materials which demonstrate the high tensile strength, lubricity, good abrasion resistance, ease of handling and good biocompatibility (Pinchuk, 1994). Shichiri *et al.* (1982) pioneered the work on needle enzyme electrodes for *in vivo* monitoring of glucose using an outer coating of polyurethane. This was a key feature of the sensor, the encapsulation in polyurethane was used as a diffusion limiting membrane in their research.

A hydrogel (MPC-co-BMA) was utilised in this chapter to encapsulate lactate enzyme electrodes to assess their function in protein solutions. The polymers, 2-methacryloyloxyethyl phosphorylcholine (MPC) and *n*-butyl methacrylate(BMA) have been copolymerised to form a hydrogel (abrieviated to MPC-co-BMA). This copolymer has an extremely hydrophilic surface with the same polar groups of phospholipid molecules and amphiphilic molecules as biomembranes, and it is considered that it has an affinity for phopholipids (Ishihara *et al.*, 1990a). Phospholipids tend to be adsorbed immediately on the MPC-co-BMA surface from a protein rich plasma solution and a stable adsorbed layer with a biomembrane-like structure is formed (Ishihara *et al.*, 1990a). It has been shown that proteins and blood cells do not adsorb onto the surface of membranes with a high MPC fraction due to weak interactions between the surface and the protein molecules (Ishihara, 1993). Acrylic beads coated with MPC-co-BMA had reduced thrombogenecity, urethane modified MPC-co-BMA improved the surface blood compatibility of segmented polyurethane membranes and cellulose grafted with polyMPC applied to cellulose membranes had improved blood compatibility and suppressed complement activation, compared to uncoated membranes (Ishihara *et al.*, 1990a; Ishihara *et al.*, 1995a; Ishihara *et al.*, 1995b).

Hence cellulose acetate, Nafion, polyurethane and MPC-co-BMA were tested for their ability to enhance the performance of the rhodinised-carbon screen-printed lactate sensors.

4.1.3. Aims.

The following list of the aims of this chapter are briefly discussed below:-

- extend the linear range of L-lactate detection at the sensor (using cellulose acetate);
- limit the interference from ascorbic acid (using cellulose acetate and/or Nafion);
- provide the sensor with some protection against protein adsorption (using either cellulose acetate, a polyurethane (Pellethane) or a hydrogel (MPC-co-BMA)).

The effect of membrane application, concentration and its solvent composition on the selectivity and sensitivity of the sensor response has not previously been fully addressed. Therefore a study of the concentration, thickness and solvent component of cellulose acetate membranes on lactate enzyme electrodes was carried out. To limit interference from ascorbic acid further, an assessment of Nafion membrane concentration and application was also carried out. The ink-jet printer was used to apply Nafion, allowing the transfer to mass production techniques. Several encapsulating membranes have been investigated for use with implantable devices in order to reduce blood passivation and increase the biocompatibility of the device. The most common are polyurethane, cellulose acetate and poly(vinyl chloride) (Lelah & Cooper, 1986). The protection against protein adsorption provided by a novel polymer, MPC-co-BMA, was assessed alongside polyurethane, and cellulose acetate membranes, to evaluate the membrane permeability under conditions similar to a biological environment.

4.2. EXPERIMENTAL.

4.2.1. General Reagents.

General reagents were used as previously discussed in Section 2.2.1.

4.2.2. Membrane materials.

The following is a list of membrane materials and the solvents in which they were dissolved:-

- Cellulose acetate (acetyl content approximately 40% from Sigma Chemical Company, London) in acetone (99% purity) and acetone-cyclohexanone mixtures.
- Nafion (perfluorinated ion exchange powder, hydrogen ion form, 5 wt% solution in a mixture of lower aliphatic alcohols and 10% water from Aldrich Chemical Company, Gillingham, Dorset) in ethanol (95% purity) and ethanol-buffer mixtures.
- A copolymer of 2-methacryloyloxyethyl phosphorylcholine (MPC) and *n*-butyl methacrylate (BMA) (MPC-co-BMA, MPC mole fraction 0.30 was a gift from Dr. K. Ishihara, Institute for Medical and Dental Engineering, Tokyo Medical and Dental University, Japan) in ethanol (99% purity).
- Polyurethane (Pellethane, 2363-80AE was kindly provided by Dr. D. Pfeiffer, Bio Sensor Technologie, Berlin, Germany) in 98% tetrahydrofuran (99%, without stabiliser) and 2% N,N'-dimethyl formamide.

Concentrations referred to in the text are percentage weight for volume.

4.2.3. Dip Coating method.

This was a very simple method of membrane application. An electrode to be encapsulated was immersed briefly into a solution of membrane material. The electrode was lightly touched on the side of the solution vessel to remove any excess solution and then left in a vertical position to dry. The vertical position ensures that the membrane dries as an even layer over the electrode surface due to the force of gravity pulling the solution to the bottom of the electrode.

4.2.4. Pipetting method.

An appropriate volume of membrane solution was drawn into a pipette (Pipetteman by Gilson, supplied by Anachem Bioscience, Anachem Limited, Luton, Bedfordshire) and then

applied evenly to the electrode surface. The electrode was left in a horizontal position to prevent the spread of solution.

4.2.5. Ink-Jet Printing.

Nafion stock solution (Aldrich Chemical Company, Gillingham, Dorset) was dissolved in 1:1 sodium phosphate buffer (0.1 M, pH 7.0 plus 0.1 M KCl) to ethanol; the buffer salts imparting electrical conductivity. Nafion solutions were then printed onto rhodinised carbon layer of an eight electrode screen printed array by means of an ink-jet printer (Biodot, Diddington, UK) which is illustrated in Figure 4.1. Such a printer operated by forcing fluid under pressure (typically 3 bar) through a small nozzle (in this case, 75 μm). As the fluid passed through the print head, an oscillating piezoelectric crystal actuated a drive rod to break the stream into fine droplets, at approximately 64,000 s^{-1} (depending on the modulation potential). An electrostatic field produced by two parallel, high voltage deflector plates caused charged droplets (charged by means of a charge electrode) to be deflected from the main jet. As the substrate passed under the print head, fluid was deposited in a controlled manner in an array of 5 x 18 droplets, completely covering the electrode working surface. This was controlled by a microprocessor, and the printed pattern was constructed in a dot matrix format. The total volume of Nafion solution deposited in one pass of the print head was 0.207 μl.

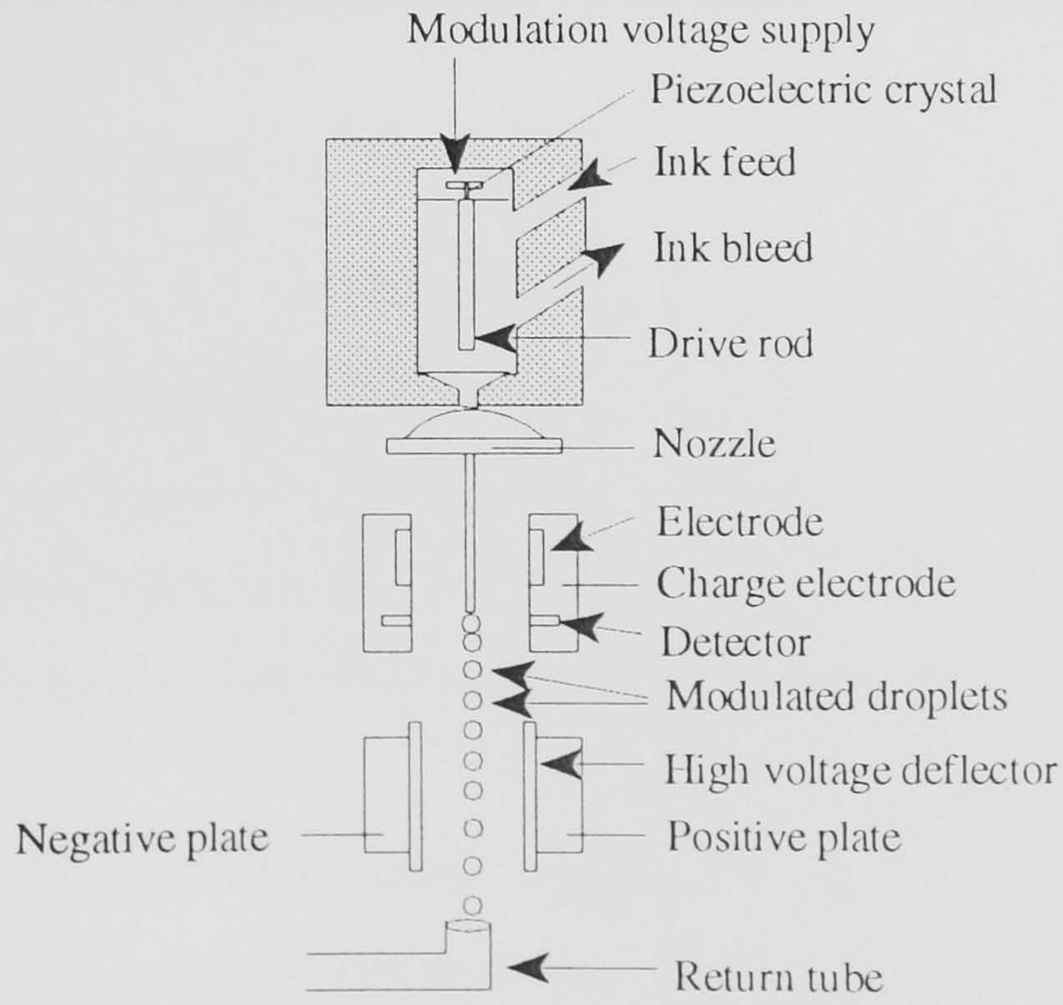


Figure 4.1. Schematic diagram of the print head of an ink-jet printer.

4.2.6. Amperometric Calibrations.

As described in Section 3.2.5.

4.2.7. Examination of Antifouling Membrane.

Individual electrodes from an eight electrode array printed with rhodinised carbon and lactate oxidase (see section 5.2.x) were dip coated with either polyurethane (4% w/v in tetrahydrofuran and N,N'-dimethyl formamide), MPC-co-BMA (0.5% w/v in ethanol) or cellulose acetate (2% w/v in acetone) and dried vertically for 30 minutes at room temperature. They were then stored with desiccant at 4°C for at least 8 hours before testing commenced. Bovine serum albumin (BSA, 98-99% Albumin, essentially fatty-acid free, Sigma Chemical Company, London) was added to phosphate buffered saline (PBS, 0.1 M potassium phosphate, pH 7.4, plus 0.15 M sodium chloride) to give a concentration of 4.5 $g.L^{-1}$ (BSA.PBS), equivalent to the protein concentration in plasma (Long *et al.*, 1971). After three calibrations with L-lactate had been performed on the electrode of interest in PBS, three more calibrations were carried out in BSA.PBS, after immersion in the protein solution for 5 minutes. The electrode was then stored in BSA.PBS for 18 hours, after which it was again calibrated three times with L-lactate in plain PBS.

4.2.8. Measurement and Presentation of Data.

Measurement and presentation of data were carried out as previously mentioned in Section 2.2.20.

4.3. RESULTS.

4.3.1. Cellulose Acetate Investigation.

Cellulose acetate membranes were used to extend the linear range of lactate detection by rhodinised carbon and lactate oxidase screen-printed electrodes (abbreviated as RhCLODSPE). By altering the cellulose acetate concentration of the solution applied to the sensors, the lactate detection characteristics changed (see Figure 4.2.). A high cellulose acetate concentration afforded a sensor with a large linear range but low sensitivity. Conversely, low cellulose acetate concentrations in the membrane provided little extension of the linear range but the sensitivity to L-lactate was much greater. Cellulose acetate (2% w/v in acetone) allowed the sensors to detect L-lactate linearly in the range 0 – 3 mM with a sensitivity of 2.55 $\mu A.mM^{-1}$ L-lactate. The coefficient of variation of four electrodes at 1 mM L-lactate was 30%.

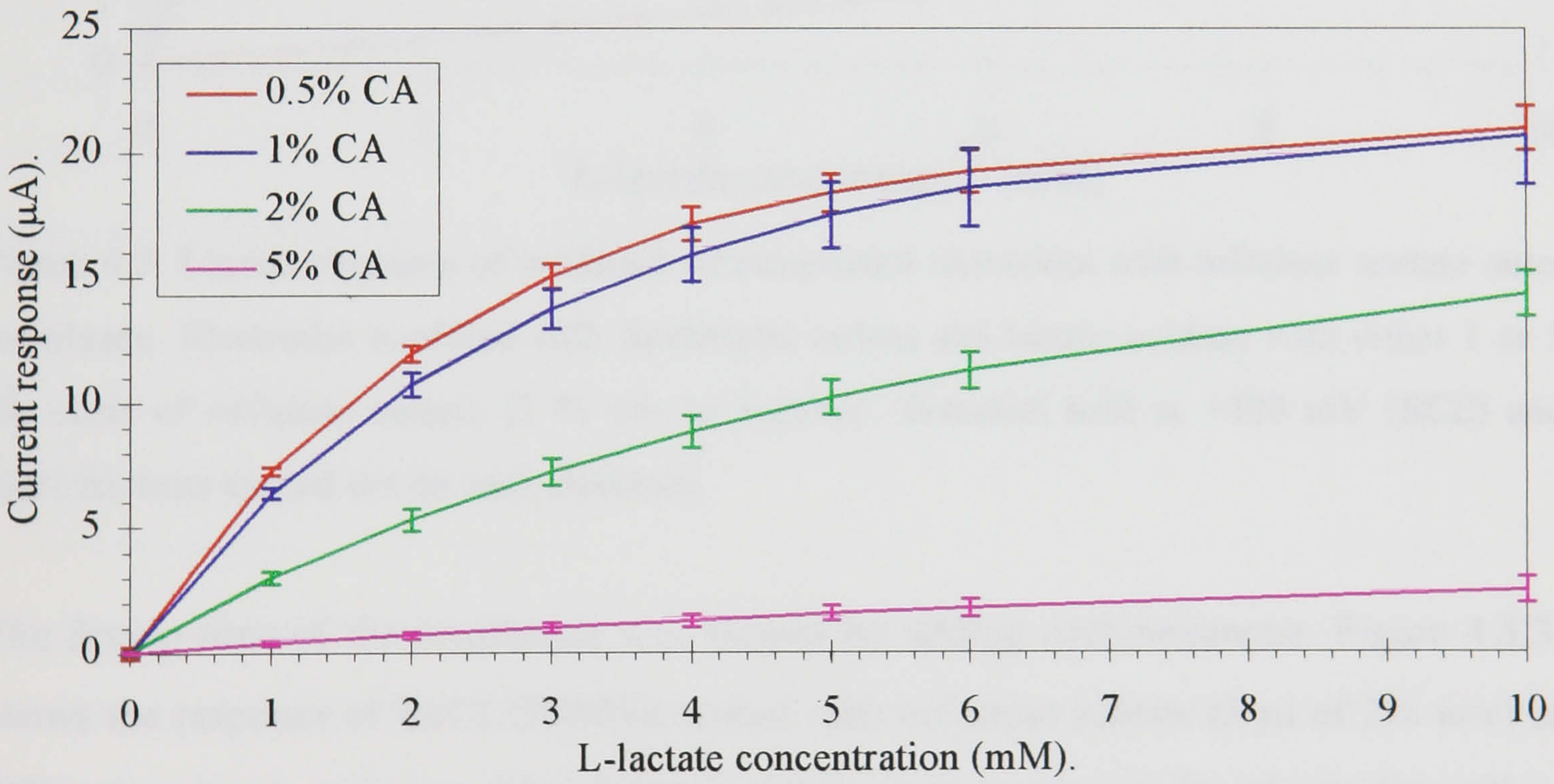


Figure 4.2. Response to lactic acid by modified screen-printed electrodes with cellulose acetate outer membranes. Electrodes modified with rhodinised carbon and lactate oxidase inks and then aliquots of cellulose acetate solutions (3 µl of either 0.5, 1, 2 or 5 % w/v in acetone) applied to the surface. Potential held at +400 mV (SCE) and four electrodes tested in triplicate.

The number of membrane layers applied to the electrode also altered the sensor response. The dip coating method was used to form membranes with 2% cellulose acetate content onto RhCLODSPE. These were tested with L-lactate at +400 mV (SCE)

and the results are shown in Figure 4.3. It can be seen that the sensor with more cellulose acetate layers gave a larger linear range but lower sensitivity. The linear ranges and sensitivities toward L-lactate of the sensors with 1 and 5 coats of 2% cellulose acetate was 0 – 1.2 mM and 0 – 10 mM, and 5.26 $\mu A.mM^{-1}$ and 0.22 $\mu A.mM^{-1}$ respectively. The coefficient of variation at 1 mM L-lactate was 11% and 5% for 1 and 5 coats, respectively.

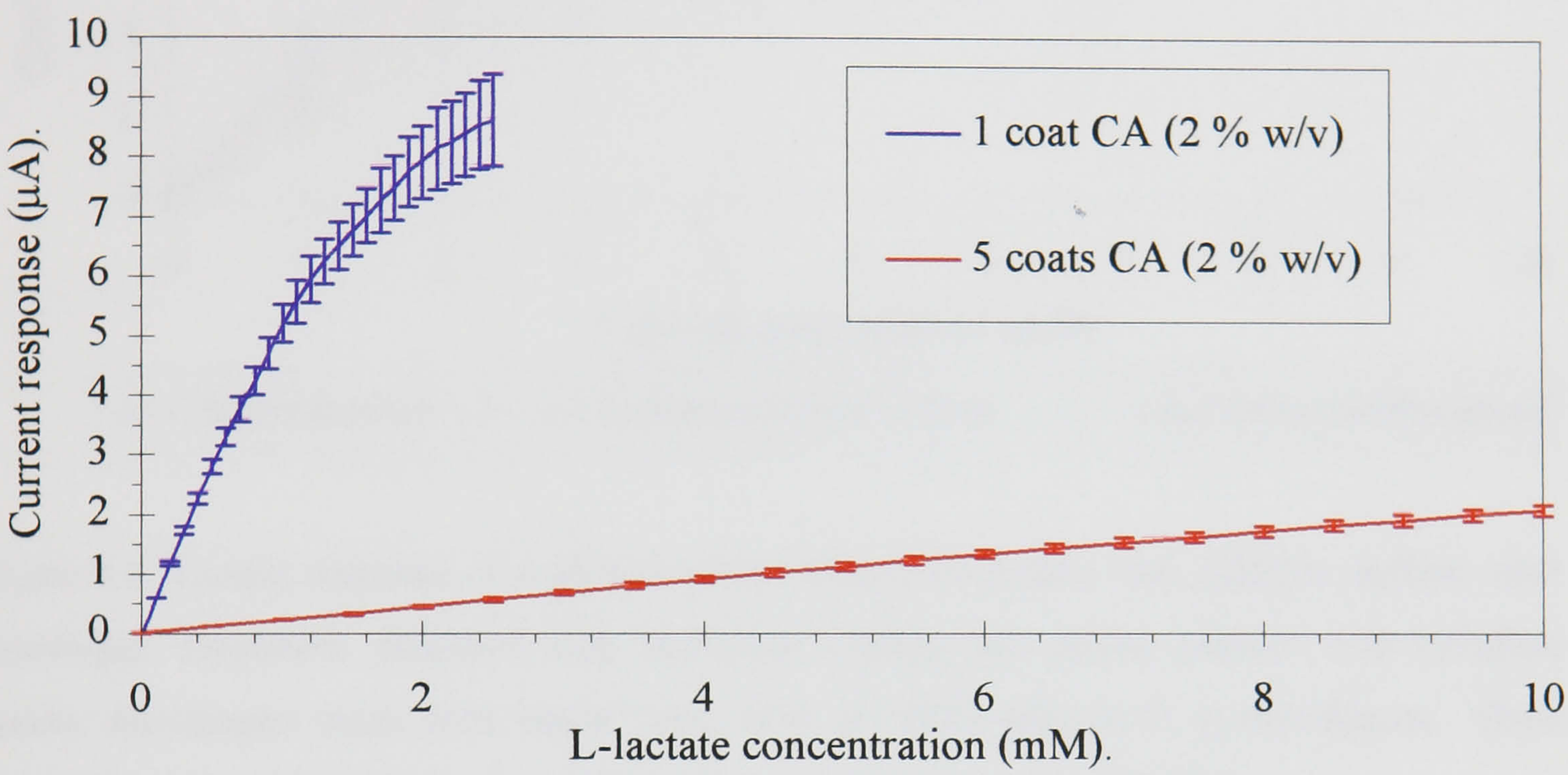


Figure 4.3. Lactate response of modified screen-printed electrodes with cellulose acetate outer membrane. Electrodes modified with rhodinised carbon and lactate oxidase with either 1 or 5 dip coats of cellulose acetate (2 % w/v in acetone). Potential held at +400 mV (SCE) and triplicate tests carried out on each electrode.

The drying time of the membrane was slowed by adding cyclohexanone. Figure 4.3.3. shows the response of RhCLODSPEs coated with cellulose acetate (3 µl of 2% w/v) in different solvent mixtures. The dynamic range to L-lactate of the electrodes with a membrane of 1:1 acetone to cyclohexanone was extended as well as performing more linearly than the electrodes with a membrane of 100% acetone. The response to lactate of the 1:4 acetone to cyclohexanone electrodes was lowered although the dynamic range was not extended compared to the other sensors. By adding cyclohexanone, the calibration plot to ascorbate was more curved, less linear, as well as being less sensitive (results not shown).

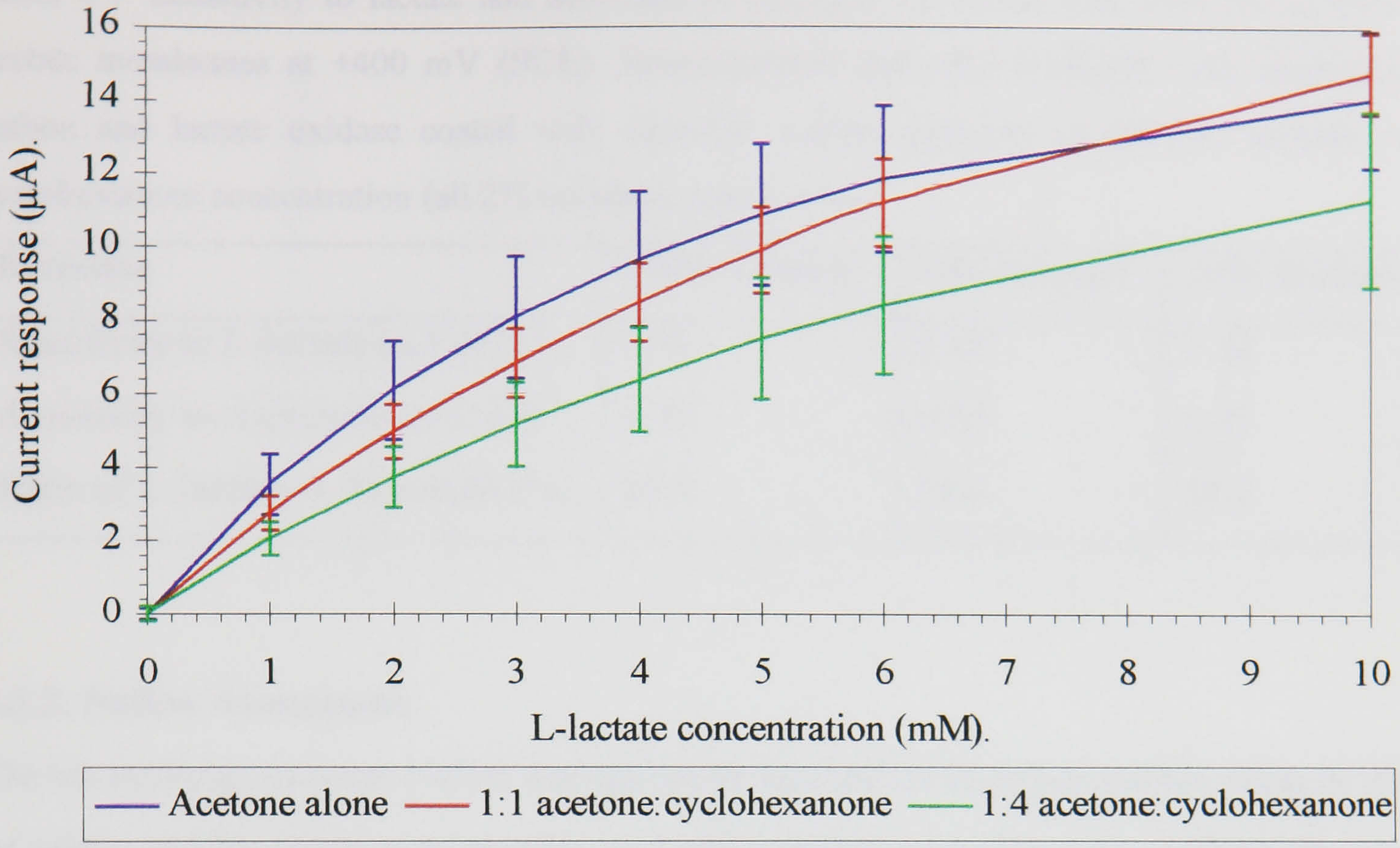


Figure 4.4. Lactate response of modified screen-printed electrodes with cellulose acetate outer membrane. Electrodes modified with rhodinised carbon and lactate oxidase with cellulose acetate membranes made with either 20%, 50% or 100% acetone in cyclohexanone. Three electrodes with each membrane were tested (in triplicate) at +400 mV (SCE).

Table 4.1. shows the sensitivity to L-lactate and ascorbate and the ratio of these responses from RhCLODSPE coated with cellulose acetate (3 μl, 2% w/v) made in different solvents. The responses of the electrodes with a membrane containing 1:4 acetone to cyclohexanone content gave a higher lactate to ascorbate ratio than the other sensors but the sensitivity of the L-lactate detection was not as great. The membrane containing 1:1 acetone to cyclohexanone provided a larger dynamic range to lactate and a lowered sensitivity to ascorbate at higher concentrations, compared to the membrane containing 100% acetone. The coefficient of variation of response to 1 mM L-lactate at electrodes coated with cellulose acetate (2% w/v) in 1:0, 1:1 and 1:4 acetone to cyclohexanone were 41%, 29% and 39% respectively.

Table 4.1. Sensitivity to lactate and ascorbate of modified electrodes with different cellulose acetate membranes at +400 mV (SCE). Screen-printed electrodes modified with rhodinised carbon and lactate oxidase coated with cellulose acetate solutions of different acetone in cyclohexanone concentration (all 2% cellulose acetate w/v).

Response	100% Acetone	50% Acetone	20% Acetone
Sensitivity to L-lactate ($\mu A.mM^{-1}$)	2.39	2.27	1.72
Sensitivity to Ascorbate ($\mu A.mM^{-1}$)	9.79	9.20	6.07
Ratio of L-lactate to Ascorbate (%)	24.4	24.6	28.2

4.3.2. Nafion Assessment.

The ion exchange polymer Nafion was applied to the rhodinised-carbon surface using an ink jet printer and the response to ascorbic acid and hydrogen peroxide of these electrodes was assessed, along with other methods of application and a cellulose acetate membrane. The results show two findings: the effect of Nafion concentration and the effect of the method of application on the electrode response. The response to 1 mM hydrogen peroxide and to 0.28 mM ascorbic acid and the corresponding ratios are shown in Figure 4.6.

Electrodes with no rhodinised carbon ink showed a reduced response from interference when a Nafion membrane was applied. The response to 5 mM hydrogen peroxide before and after a Nafion membrane was applied was 0.01 μA whereas the response to 0.28 mM ascorbic acid was 1.16 μA and 0.18 μA. This means that the ascorbic acid response was decreased by 85% whereas the hydrogen peroxide response was not decreased in the presence of a Nafion layer. As a general trend, however, the addition of Nafion lowered the hydrogen peroxide response and can be seen prominently at thicker membrane layers. Electrodes E, M and O were coated with five sets of ten passes of Nafion, increasing in concentration from 0.5% to 1% to 2%. It can be seen from Figure 4.6. that the hydrogen peroxide response decreases as the Nafion concentration increases. The hydrogen peroxide response is greatest at electrodes with 0.1% Nafion (C) and those with 1% Nafion applied by ten passes of the ink-jet printer (I). However, the most repeatable responses (electrodes which gave the smallest error) were electrodes ink-jet printed with five sets of ten passes with 0.5% Nafion (E) or five sets of ten passes with 1% Nafion (M).

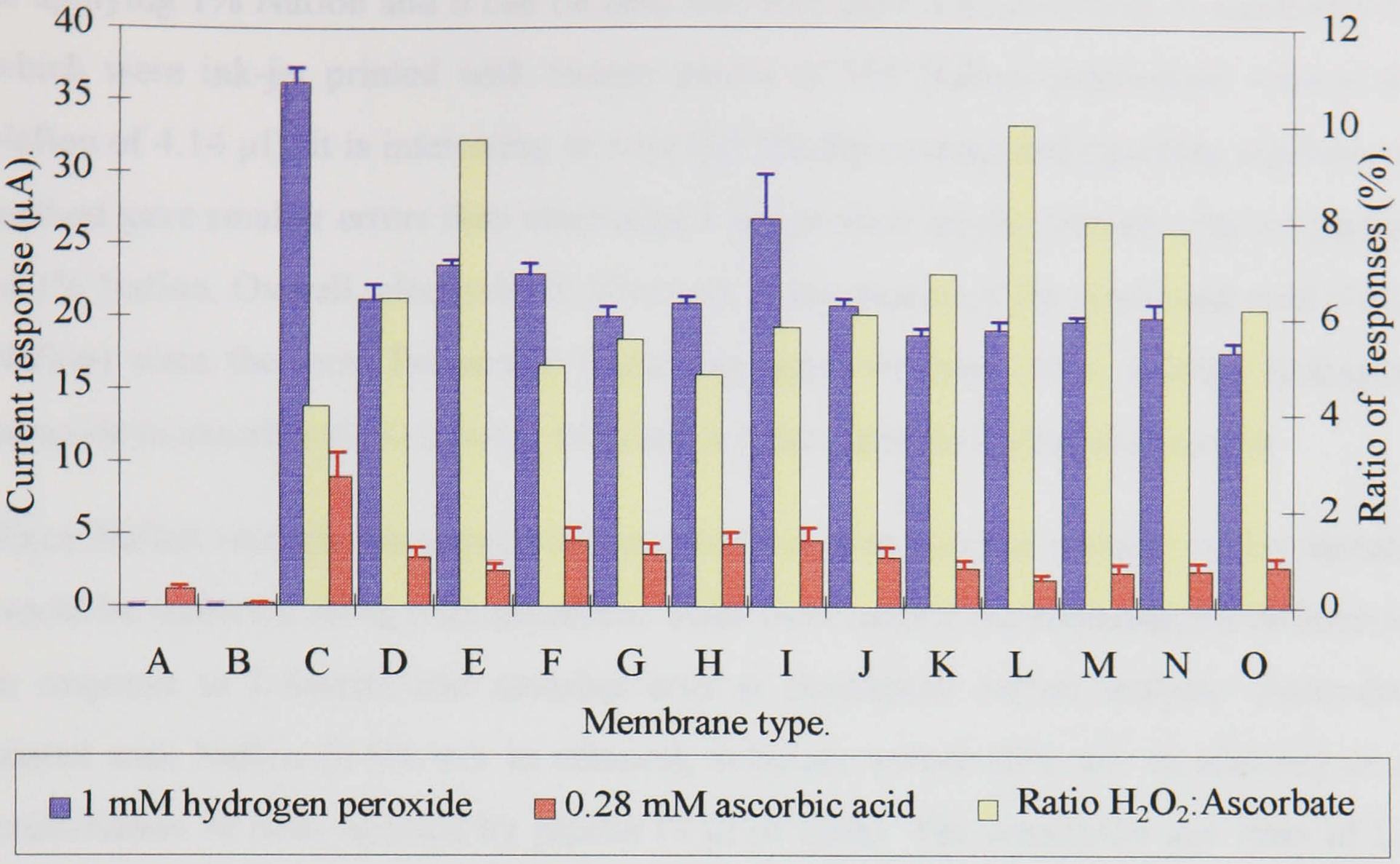


Figure 4.5. Response to hydrogen peroxide and ascorbic acid of rhodinised carbon screen-printed electrodes coated with Nafion or cellulose acetate at +400 mV (SCE). Three electrodes tested in triplicate. The following list is the membrane application method for each electrode:

A	Carbon pad, no membrane	H	Ink-jet 20x1 1% Nafion
B	Carbon pad, dip coated in 1% Nafion	I	Ink-jet 10x1 1% Nafion
C	Pipetted 2.5 µl 0.1% Nafion	J	Ink-jet 10x2 1% Nafion
D	Ink-jet 10x2 0.5% Nafion	K	Ink-jet 10x3 1% Nafion
E	Ink-jet 10x5 0.5% Nafion	L	Ink-jet 10x4 1% Nafion
F	Dip coated 1% Nafion	M	Ink-jet 10x5 1% Nafion
G	Pipetted 5 µl 1% Nafion	N	Ink-jet 10x2 2% Nafion
		O	Ink-jet 10x5 2% Nafion

By comparing the ascorbic acid response at electrodes D with J and N, and E with M and O it can be seen that increasing the concentration of Nafion did not affect the ascorbic acid response although increasing the layer thickness did reduced it slightly. The lowest response to ascorbic acid was with the electrodes that were ink-jet printed with five sets of ten passes of 0.5% Nafion (E) and four or five sets of ten passes of 1% Nafion (L, M). The greatest ratio of hydrogen peroxide to ascorbic acid response was gained at electrodes ink-jet printed with five sets of ten passes of 0.5% Nafion (E) or four sets of ten passes of 1% Nafion (L). Electrodes F and G were alternative methods

of applying 1% Nafion and it can be seen that they gave similar results to electrodes H which were ink-jet printed with twenty passes of 1% Nafion (equivalent volume of Nafion of 4.14 μl). It is interesting to note that the dip-coating and pipetting application method gave smaller errors than electrodes I which were ink-jet printed with ten passes of 1% Nafion. Overall, electrodes E (five sets of ten passes of the print head with 0.5% Nafion) were the most favourable since they gave the least error, a large hydrogen peroxide to ascorbic acid response ratio and a large signal to hydrogen peroxide.

Since Nafion restricts the passage of anions, there was concern whether or not lactate would be restricted along with ascorbate. Tests were carried out assessing the difference in response to L-lactate and ascorbic acid at rhodinised carbon enzyme electrodes coated with Nafion (0.5% w/v in ethanol), cellulose acetate (2% w/v in acetone) or a combination of both, applied by pipette (3 μl of each). The sensitivity and ratio of L-lactate to ascorbate response are shown in Figure 4.7. The slope from 0 – 3 mM L-lactate and 0 – 1 mM ascorbic acid were calculated. The electrodes coated with both Nafion and cellulose acetate were linear up to 4 mM L-lactate but neither the cellulose acetate or Nafion coated electrodes gave a linear response to L-lactic acid. All sensors detected L-lactate up to 10 mM although the sensor coated with cellulose acetate alone gave a higher response than the other electrodes and could detect higher concentrations. The coefficient of variation at 1 mM L-lactate was 30%, 32% and 58% for electrodes coated with cellulose acetate, Nafion and both membranes, respectively. All sensors gave linear responses to ascorbic acid.

It can be seen that although enzyme electrodes coated with cellulose acetate or Nafion alone had the same sensitivity towards L-lactate, the sensitivity to ascorbic acid was much greater at the Nafion coated enzyme electrode. This is illustrated by the drop in the ratio of L-lactate to ascorbic acid response at the Nafion alone coated enzyme electrodes. When both Nafion and cellulose acetate were applied to the enzyme electrodes, even though the sensitivities to both L-lactate and ascorbic acid had dropped considerably, the ratio of these sensitivities had increased over that obtained with the electrode coated with Nafion alone.

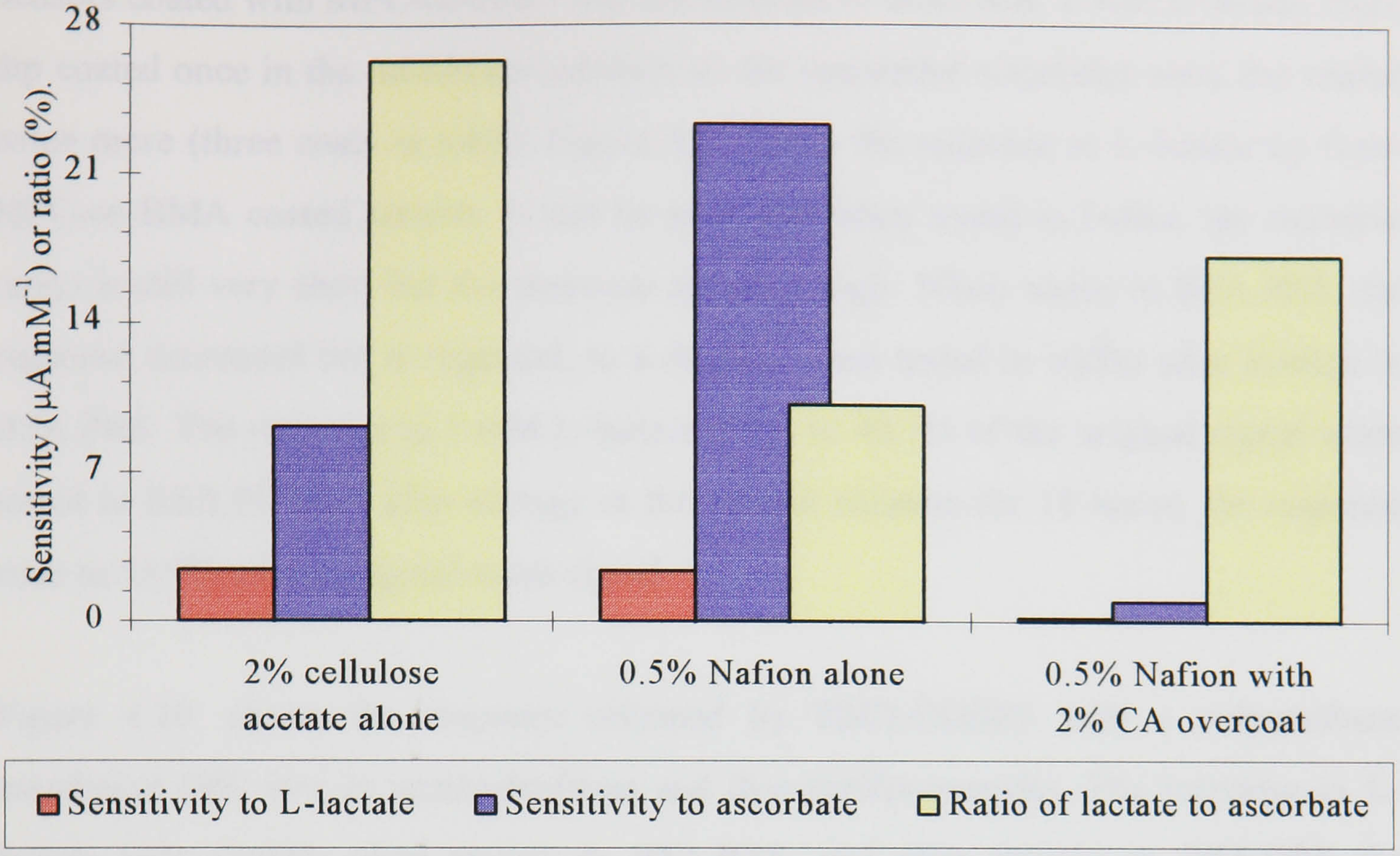


Figure 4.6. Responses to lactate and ascorbate of modified screen-printed electrodes with different membranes at +400 mV (SCE). Electrodes modified with rhodinised carbon and lactate oxidase and coated with either Nafion, cellulose acetate or a combination of both.

4.3.3. Examination of Anti-fouling Membranes.

An investigation into the anti-fouling properties of polyurethane, MPC-co-BMA and cellulose acetate was carried out. The rhodinised carbon, lactate oxidase screen-printed electrodes (RhCLODSPE) were encased with membrane by dip coating once in a solution of relevant concentration. A response to L-lactate was carried out at +400 mV (SCE) in buffer (PBS), then buffer containing bovine serum albumin (BSA.PBS) and then stored in this solution for 18 hours before testing once more in buffer.

RhCLODSPE with cellulose acetate membranes responded well to L-lactate, as can be seen in Figure 4.8. When tested in BSA.PBS, the mean current response dropped slightly to 84.1% of the original signal (taken at 5 mM L-lactate). When retested in buffer after storage in BSA.PBS for 18 hours, the mean current response was significantly lower, only 54.6% of the original response at 5 mM L-lactate.

Sensors coated with MPC-co-BMA did not respond to more than 2 mM L-lactate when dip coated once in the membrane solution so the remainder electrodes were dip coated twice more (three coats in total). Figure 4.9. shows the response to L-lactate by these MPC-co-BMA coated sensors. It can be seen that when tested in buffer, the dynamic range is still very short but the response signal is high. When tested in BSA.PBS, the response decreased but is regained, to a degree, when tested in buffer after storage in BSA.PBS. The response to 5 mM L-lactate drops to 40.7% of the original signal when tested in BSA.PBS but after storage in the protein solution for 18 hours, the response rises to 53.7% of the original mean signal.

Figure 4.10. shows the response obtained by RhCLODSPE with a polyurethane membrane (4% w/v in tetrahydrofuran and dimethylformamide). The response to L-lactate falls slightly upon testing in BSA.PBS, and after storage in BSA.PBS the response falls slightly more. The mean signal to 5 mM L-lactate decreases from 100% to 92.0% to 80.3% when tested successively in PBS, BSA.PBS and PBS (after storage in BSA.PBS for 18 hours), respectively.

Overall, the polyurethane coated sensor response decreased the least upon testing and storage in BSA.PBS. The dynamic range of all sensors was lowered once exposed to the bovine serum albumin, although the trend is more obvious at the MPC-co-BMA coated sensors. However, the MPC-co-BMA coated sensors did provide the greatest signal to L-lactate whereas polyurethane coated sensors gave the most linear response and the lowest signal to L-lactate, and cellulose acetate coated sensors gave a high signal and wide dynamic range.

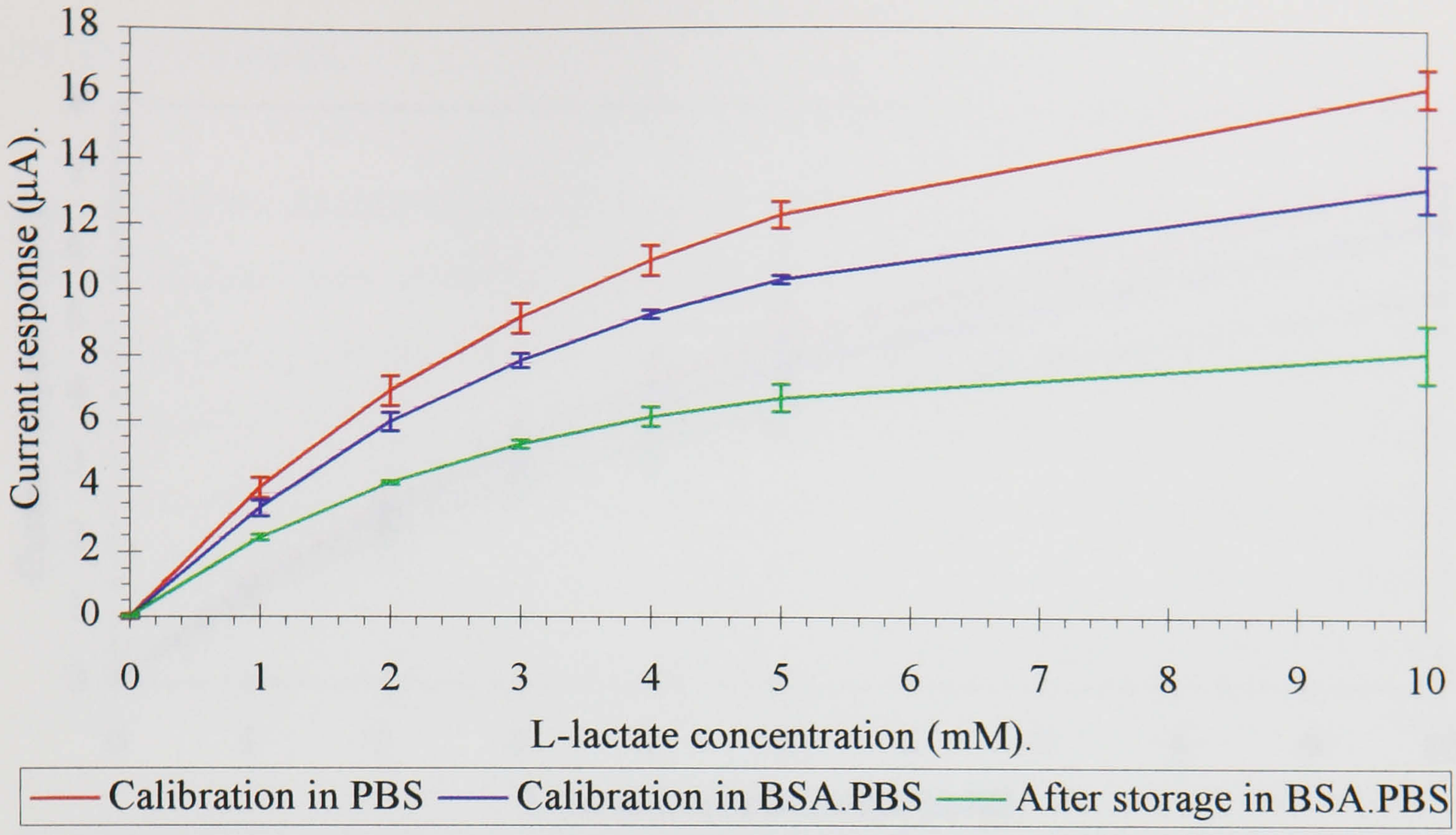


Figure 4.7. Current response to lactic acid of modified screen-printed electrode with a cellulose acetate coating. Three electrodes modified with rhodinised carbon and lactate oxidase with a cellulose acetate (2 % w/v in acetone) outer membrane, tested in triplicate. Response measured at +400 mV (SCE) in a stirred solution of either buffer or buffer containing bovine serum albumin.

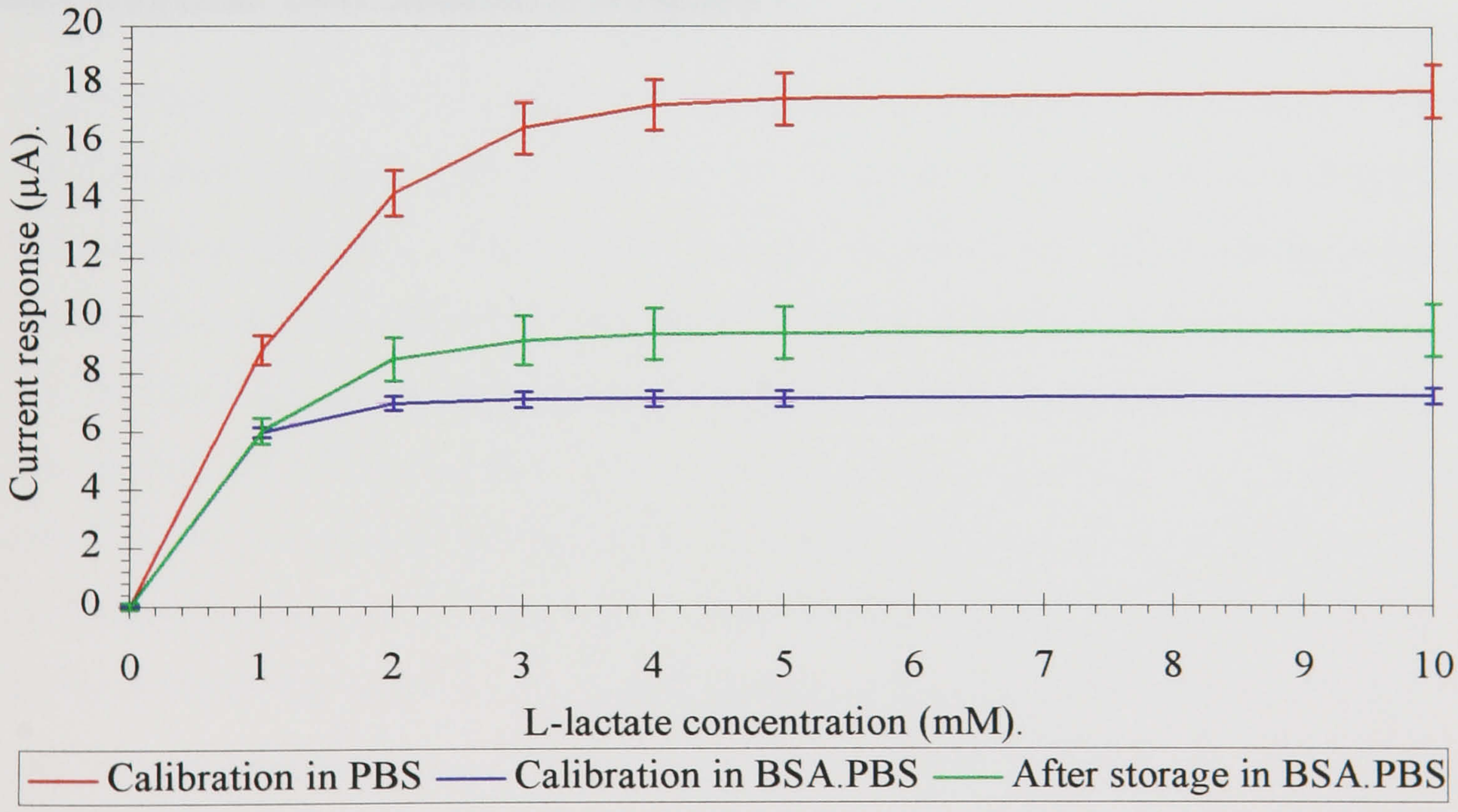


Figure 4.8. Current response to lactic acid of modified screen-printed electrode with a MPC-co-BMA coating. Three electrodes modified with rhodinised carbon and lactate oxidase with a MPC-co-BMA (0.5 % w/v in ethanol) outer membrane and tested in triplicate. Other conditions as in Figure 4.7.

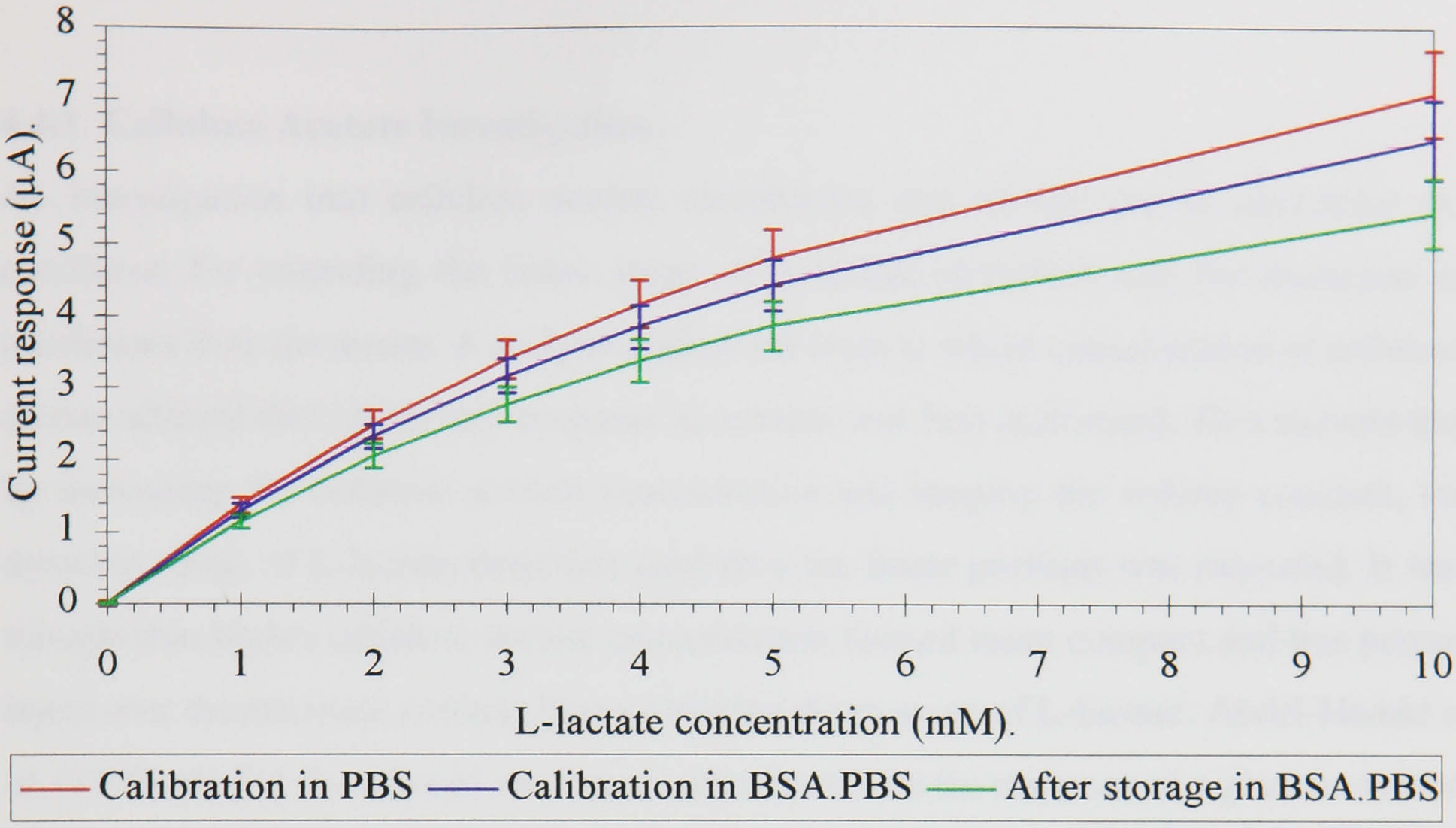


Figure 4.9. Current response to lactic acid of modified screen-printed electrode with a polyurethane coating. Five electrodes modified with rhodinised carbon and lactate oxidase with a polyurethane (4 % w/v in tetrahydrofuran and dimethyl formamide) outer membrane and tested in triplicate. Other conditions as in Figure 4.7.

4.4. DISCUSSION.

4.4.1. Cellulose Acetate Investigation.

An investigation into cellulose acetate membranes was carried out to determine the conditions for extending the linear range of L-lactate detection and the exclusion of interferents from the sensor. A study to explore the ways in which concentration of cellulose acetate affected the L-lactic acid response at sensors was first performed. This showed that by increasing the cellulose acetate concentration and keeping the volume constant, the dynamic range of L-lactate detection (and thus the linear portion) was extended. It was thought that higher cellulose acetate concentration formed more compact and less porous layers over the electrode surface, thus restricting the passage of L-lactate. Abdel-Hamid *et al.* (1995) studied the affect of membrane concentration on the response of a glucose sensor. Although they used the dip coating method of membrane application, which would not result in a consistent membrane thickness, they also found that higher cellulose acetate concentrations widened the linear range. In addition, the sensitivity to glucose decreased and the response time increased. Pfeiffer *et al.* (Pfeiffer *et al.*, 1992) also demonstrated that with a material of constant thickness, a significant increase in linear range was obtained by a diminished porosity and not simply by a reduction in pore diameters. As a result of higher cellulose acetate concentrations, however, the sensitivity was decreased and the response time increased, therefore a compromise was sought. If a membrane of low cellulose acetate concentration was made thicker, it would form a wider diffusion layer and thus limit the rate at which L-lactate diffuses through the membrane. This was illustrated by using a series of dip coats of 2% cellulose acetate. The cosubstrate of the enzyme reaction, oxygen, is not restricted as much as lactate as it can diffuse through the cellulose acetate matrix as well as through the pore structure (Pfeiffer *et al.*, 1992). Thicker layers of cellulose acetate on the sensor extended its dynamic and linear range of detection, but the sensitivity and response time were correspondingly lowered. Anzai *et al.* (1992) reported similar findings using a lactate sensor coated with cellulose acetate. The thickness of the membrane was increased successively by repeating the dip-coating procedure. This resulted in an increase in the dynamic range but a decrease in the sensitivity and response time.

By changing the drying time of the cellulose acetate membrane solution, it was believed that

the pore structure would be altered which could be utilised in interference exclusion as well as extending the linear range. This was based on the rationale that increasing the drying time would decrease the pore size as described by White *et al.* (1996). The molecular weight of ascorbic acid is twice that of L-lactate therefore it was reasoned that the molecular size of ascorbic acid would also be larger. The concept was to allow L-lactate to diffuse through pores small enough to exclude the ascorbic acid, hence reduce interfering signals. This is the basis for membranes with molecular-weight cut-off points. The hypothesis was examined using cyclohexanone to increase drying time and thus decrease pore size. The same volume of membrane solution was applied to each sensor surface to eliminate the risk of depositing more membrane during dip coating due to viscous solutions. The results showed that by slowing the drying time the dynamic range of lactate detection was extended and the interference from ascorbic acid was reduced although not completely eliminated. The performance of 2% cellulose acetate, weight for volume in a solution of one to one, cyclohexanone to acetone, was the best membrane in terms of sensitivity, dynamic range and ascorbic acid exclusion from the selection of cellulose acetate solutions tested. It also provided a low error in repeatability between electrodes.

4.4.2. Nafion Assessment.

The perfluorinated ion exchange powder, Nafion, was evaluated as a means of enhancing selectivity of the lactate sensors. A series of concentrations and layers of Nafion were applied over the catalytic carbon layer with the aim of printing the enzyme ink on top. Nafion is often applied adjacent to the electrode to allow the passage of low molecular weight species like hydrogen peroxide but electrostatically repel negatively charged species (Doretti *et al.*, 1994; Heineman *et al.*, 1991). A membrane had to be applied over the rhodinised carbon ink to stop the ink from dissolving in the buffer solution. However, by using the bare carbon pad the amount of ascorbic acid exclusion could be calculated. The carbon pad responded less to ascorbate upon the application of Nafion (only 15% of the original signal) but the hydrogen peroxide response was not affected. Heineman *et al.* (1991) similarly found that a thin layer of Nafion deposited directly to the graphite electrode exhibited a 96% attenuation of the ascorbate signal. Nafion reduced the electrode response to hydrogen peroxide as well as ascorbic acid at the rhodinised carbon electrodes. The Nafion membrane adds an additional diffusion barrier which lowers the flux of hydrogen

peroxide to the electrode surface, thus reducing the signal. However, the signal reduction is greater for ascorbate and other anionic species because both the electrostatic repulsion and diffusion barrier effects are combined. This was also illustrated by Pan and Arnold (1996) who reported that at +400 mV (Ag/AgCl) the response to ascorbate relative to a platinum electrode with no Nafion, decreased to 12% upon the application of a 2.2 μm Nafion membrane, and to 0.4% on the application of a 11 μm Nafion membrane. They also reported that the hydrogen peroxide response was not reduced to the same degree as ascorbic acid.

4.4.3. Method of Membrane Application.

Although the dip coating method is fast and simple, the volume of sample applied is unknown and may vary between electrodes. The material absorbency and the membrane solution viscosity play a role in the thickness of the membrane layer. The pipetting method is more reproducible in terms of volume application, although a very steady hand is needed to apply sample to the whole working surface without touching it. Too much fluid could also result in the sample flooding across the working surface and onto the substrate whereas too little means that the membrane may not cover the whole working area. Ink-jet printing was used to improve the reproducibility of membrane drop deposition. The discrepancy between electrode responses with ink-jet printed membrane was due to the speed at which the substrate passed under the print head, a faster pass resulted in highly dispersed droplets. Even though the error was similar to that obtained when the membrane was applied by dip coating or pipetting, ink-jet printing has the advantage of allowing the sensors to be mass fabricated.

4.4.4. The use of Nafion and Cellulose Acetate to Restrict the Passage of Ascorbic Acid and L-lactate.

If the Nafion was printed on top of the lactate oxidase layer, for example when the enzyme was mixed with the catalytic rhodinised carbon ink, there may be a restriction to the L-lactate which is anionic, from reaching the enzyme. A brief study was conducted to observe the limitation of L-lactate by comparing cellulose acetate with Nafion and a combination of the two membranes. The ratio of L-lactate to ascorbate was taken in order to normalise the responses. Cellulose acetate was demonstrated to restrict ascorbic acid more than L-lactate in comparison to Nafion. A combination of both Nafion and cellulose acetate membranes

actually reduced the signals dramatically, without improvement of the lactate to ascorbic acid response ratio. This would imply that Nafion restricts the passage of L-lactate thus showing effective anionic repulsion.

4.4.5. Examination of Anti-fouling Membranes.

It is well known that proteins and cells adsorb on the surface of foreign materials when they come into contact with living tissues and fluids (Lelah & Cooper, 1986). Generally, *in vivo* sensors are coated with polyurethane or cellulose acetate but there has been no investigation concerning the improvement in sensor biocompatibility (Ishihara *et al.*, 1992) and such a study would be a major undertaking. An antibody response in rats against cellulose acetate, polyurethane and regenerated cellulose coated glucose sensors was found to occur by Ziegler *et al.* (1994), although a cell culture showed only minor toxicity to the membranes. The adsorption layer of biocomponents formed during contact with biological samples interferes with the permeation of the target compound (Ishihara, 1992; Shichiri *et al.*, 1987). The use of buffer solutions containing albumin concentrations analogous to the concentration in blood allows the antifouling and permeation behaviour of membranes to be easily studied. A comparison of sensor response in buffer and albumin enriched buffer solutions was undertaken, using cellulose acetate, polyurethane and novel MPC-co-BMA membranes at concentrations cited in the literature to be effective at reducing surface fouling (Ishihara *et al.*, 1995a; Shichiri *et al.*, 1982; Sittampalam & Wilson, 1983). Unfortunately, a sensor with no membrane coating could not be tested due to the instability of the water-soluble catalytic ink in the test solutions. The protein adsorption investigation showed that not more than 50% response to L-lactate was lost over a 18 hour period.

The sensors with a cellulose acetate layer responded well to L-lactate in plain buffer, they had a wide dynamic range and high sensitivity, but when tested in albumin solution, the response at 5 mM L-lactate dropped by approximately 15% and the dynamic range was also reduced. This trend is emphasised after storage in the albumin solution, both the sensitivity and dynamic range are reduced when tested in buffer. These results indicate that irreversible protein adsorption was occurring which limited the mass transport of L-lactate to the enzyme at the electroactive surface. Sittampalam and Wilson (1983) used cellulose acetate to coat a platinum electrode at which they measured hydrogen peroxide concentrations in

plain and albumin containing solutions. They recorded only a very small reduction in sensitivity at a platinum electrode coated with cellulose acetate film (2% in 1:1 cyclohexanone to acetone solution) in the presence of albumin (200 mg.dL^{-1}), compared to a large drop in response when the cellulose acetate film was absent. Sittampalam and Wilson suggested that poisoning of the platinum electrode, resulting from protein fouling, was prevented by the cellulose acetate film. The bovine serum albumin concentration used by Sittampalam and Wilson was much lower than the physiological concentration (4.5 g.dL^{-1}). It can therefore be assumed that protein adsorption would be greater at physiological concentrations, although the electrode poisoning would still be prevented since albumin (Mr 60,000) is too large to diffuse through the film (Sittampalam & Wilson, 1983).

The MPC-co-BMA membrane allowed the sensor to sensitivity detect L-lactate, but it was too thin to provide a wide dynamic range. The drop in response of lactate enzyme electrodes coated with MPC-co-BMA, while disappointing, shows an interesting feature. Even though the response to 5 mM L-lactate drops to 40% in albumin solution from plain buffer, approximately 13% of the response was actually recovered after storage in albumin solution and then retesting in plain buffer. The dynamic range was also slightly regained. Since the response recovered slightly after storage and washing, this discounts the possibility that the initial drop in response was entirely due to denaturing of the lactate oxidase. MPC-co-BMA hydrates in water but does not dissolve, therefore loss of membrane does not occur (Ishihara *et al.*, 1990b). However, the enzyme may seep into solution very slowly because the MPC-co-BMA allows slow permeation of bovine albumin (M_r 60,000) and, although this is slightly smaller than lactate oxidase (M_r 80,000), permeation is only prevented with very large molecules like bovine γ-globulin (M_r 150,000) (Ishihara *et al.*, 1990b). This indicates that leeching of lactate oxidase may be partially responsible the drop in the initial response. The recovery of the enzyme electrode response most probably occurs because protein adsorption is reversible on the MPC-co-BMA. Albumin physically coats the surface but does not form strong chemical bonds enabling albumin to be washed off in aqueous solution. This can be explained because MPC-co-BMA has a very hydrophilic surface which does not cause the albumin molecules to unfold to reveal any hydrophilic amino acids (as discussed in Section 4.1.1.). Thus only hydrogen bonding and weak van der Waals forces keep the albumin-MPC-co-BMA interface in place. Ishihara (1993) demonstrated that there was a

weak interaction between the MPC-co-BMA surface and proteins.

L-lactate enzyme electrodes coated with polyurethane have a more linear response to L-lactate than the other coated sensors, but the sensitivity was also much reduced (approximately half that of the cellulose acetate coated electrodes). This indicates that polyurethane limits the mass transport of L-lactate to the electrocatalytic surface. Churchouse *et al.* (1986) tested polyurethane membranes on needle lactate enzyme electrodes and the linear range was extended from 0 – 0.3 mM to 0 – 7 mM for an electrode before and after dip coating in polyurethane (4% w/v). They showed by electron microscopy that polyurethane dip-coated directly onto needle electrodes had a microporous structure which allowed the passage of L-lactate, thus creating a diffusion limiting membrane (Churchouse *et al.*, 1986). Shichiri *et al.* (1982) used polyurethane membranes to extend the linear detection range to glucose of needle enzyme electrodes.

The resistance of polyurethane to protein adsorption is clear, only 20% of the signal to L-lactate is lost over the course of the test. This indicates that either albumin adsorbed irreversibly to the polyurethane surface or the enzyme activity of the sensor had dropped. This is reasoned because, unlike MPC-co-BMA coated sensors, the response was not regained after washing in plain buffer. Similar results were obtained by Shichiri *et al.* (1982). A polyurethane coated needle glucose sensor was reported to loose only approximately 20% sensitivity when tested in 7% albumin solution for 2 hours at 37°C, with 5% oxygen (Shichiri *et al.*, 1982). When implanted into dogs, the sensitivity of the needle glucose sensors gradually decreased to 81% of the initial level over 3 days' continuous monitoring (Shichiri *et al.*, 1982).

4.4.6. Conclusions.

Manipulation of cellulose acetate membrane concentration and thickness attenuated the ascorbic acid interference. By controlling the thickness of cellulose acetate films, the dynamic range of L-lactate detection, and hence linear range, was extended without increasing the error of response at lactate oxidase and rhodinised carbon screen-printed electrodes. It has been shown that although Nafion limited the ascorbic acid, the passage of L-lactate to the underlying electrode surface was also restricted. Nafion can not therefore be used as an outer membrane of sensors for L-lactate.

Although ink-jet printing was applied to drop-coat Nafion solution in an automated fashion, the manual manner in which the print head was propelled accross the electrode reduced the reproducibility of the membrane layer. An alternative method of solution deposition was later explored, a fully automated drop-on-demand device called a Cavro printer.

The protein adsorption test illustrated that polyurethane would be suitable as an outer membrane coating for operation of an amperometric L-lactate enzyme electrode in blood. These findings are in agreement with those of Churchouse (1986) who found the performance of poly(hydroxyethly methacrylate), cellulose acetate, silicone rubber, nylon and various silanes to be unsatisfactory as an outer coating for a needle-type device because of either signal attenuation or poor linearity. A preliminary study shows polyurethane to be a favourable outer membrane, but its concentration and application with the improved cellulose acetate membranes also needs to be investigated. The copolymer MPC-co-BMA shows promise as an anti-fouling if it could provide greater stability of the electrode response. This would require further study of the formulation in relation to the membrane permeability.

CHAPTER 5:

SENSOR IMPROVEMENT AND ASSESSMENT.

5.1. INTRODUCTION.

This chapter deals with the alterations made to the fabrication process of the rhodinised carbon L-lactate sensors to improve their operation and performance. Development of implantable and *in vitro* sensing devices to the stage of commercialisation is hindered by problems of fabrication and fouling. Many researchers hand fabricate devices which is time consuming, expensive and does not achieve the reproducibility desired. Chapter 4 investigated the use of a novel co-polymer (MPC-co-BMA), cellulose acetate and polyurethane as sensor coatings to improve the resistance to surface fouling by blood proteins. The issues of surface fouling, heamocompatibility and biocompatibility have also been addressed by many other workers, and the formulation of an ideal membrane structure has still not been discovered. This chapter approaches these problems in an empirical manner, disposable sensors were produced wholly by mass fabrication techniques and the membrane structure adapted to the use which was required by the sensor operation. Improvements to the sensor's ink formulation, based on the results from Chapter 3, design and membrane structure were studied. A flow injection system was used to evaluate the performance of the optimised sensor in terms of lactate determination within buffer, plasma and blood and the results were compared to a commercial diagnostic enzyme assay.

5.1.1. Intended Use.

The device to be constructed was for use either *in vivo*, *ex vivo* or *in vitro* for the measurement of blood L-lactate. For *in vivo* monitoring, a small device has to be constructed for use within a blood vessel, if it was to provide continuous monitoring of L-lactate levels. Miniature lactate probes for insertion into a blood vessel have frequently been described for potential L-lactate monitoring (Battersby & Vadgama, 1988; Churchouse *et al.*, 1986; Gorton *et al.*, 1996; Marzouk *et al.*, 1996; Mullen *et al.*, 1986; Urban *et al.*, 1994; Wang & Heller, 1993). However, the realisation of continuous *in vivo* L-lactate measurement has not often been mentioned (Baker & Gough, 1995; Hu *et al.*, 1993). The hand-fabricated needle-type amperometric L-lactate sensor described by Hu *et al.* (1993) was based on lactate oxidase immobilised on a platinum-iridium wire. A 20-gauge needle was used to insert the probe subcutaneously into anaesthetized rats where continuous L-lactate measurement was carried out at +600 mV (Ag/AgCl), for approximately 2 hours. *In*

vivo blood measurements were not carried out. Baker and Gough (1995) implanted a 2 mm diameter potentiostatic device through the superior vena cava into the heart of a dog, and continually recorded blood L-lactate levels over a 4 hour period. This hand-fabricated device was based on two oxygen sensors in a silicon rubber tube, one coupled with lactate oxidase so that the decrease in oxygen at this sensor could be correlated to the L-lactate present.

The performance of potential *in vivo* L-lactate sensing devices have often been evaluated using flow injection analysis (Battersby & Vadgama, 1988; Hu *et al.*, 1993; Marzouk *et al.*, 1996; Wang & Heller, 1993). Flow injection analysis (FIA) has also allowed many researchers to measure the L-lactate in small quantities of blood *ex vivo* or *in vitro*, to give real time information and requiring little sample preparation (Clark *et al.*, 1984; Mascini *et al.*, 1985; Meyerhoff *et al.*, 1993; Pfeiffer *et al.*, 1992; Schalkhammer *et al.*, 1994; Weaver & Vadgama, 1986). This technique also offers other advantages such as ease of operation, a fast response time and high precision.

5.1.2. Sensor Evaluation.

The main characteristics and performance of an amperometric enzyme electrode can be described by (Blum & Coulet, 1991; Buerk, 1993; Coulet *et al.*, 1991; Guilbault *et al.*, 1991):-

- detection limit;
- linearity;
- sensitivity;
- response time;
- recovery time;
- stability;
- storage;
- selectivity.

The detection limit is the lowest concentration of substrate which can be detected with an acceptable signal-to-noise ratio. A low detection limit will allow the sensing device to distinguish low concentrations from the background noise. Linearity is generally observed for substrate levels below the K_M of the enzyme unless a membrane has been used to increase the diffusion barrier; sensitivity corresponds to the gradient of this portion of the calibration curve (Coulet *et al.*, 1991). As previously discussed (Section 4.1. and 4.4.), an

increase in linearity has the drawback of decreasing the sensitivity and a compromise should be sought. The steady-state response time is given by the time required for the current response to reach a plateau after the substrate has been added, and is generally longer for sensors coated with membrane layers. The time necessary for the current to drop back to the baseline after the substrate has been washed away and the device is ready for another measurement, is known as the recovery time. Electrode aging, expressed as stability time, occurs continually and is determined to a large extent by the enzyme loading (Scheller *et al.*, 1991). The enzyme gradually becomes denatured but the rate at which this occurs can be reduced by chemical or physical stabilisers and immobilising the enzyme (Guilbault *et al.*, 1991). The measuring capability of the device may not be affected if it is frequently calibrated and the linear range may be maintained with just a small drop in sensitivity (Coulet *et al.*, 1991). Frequent calibration may also take baseline drift into consideration (Buerk, 1993). Enzyme electrodes may require special storage conditions, some may require storage at 4°C, others may still be active if kept at room temperature and some store better under dry conditions whereas others should be kept in buffer. The capability of the device to selectively detect the analyte of interest is important so that interfering signals do not elevate the response signal. If interference due to electroactive species is problematic then either a membrane or a compensating electrode strategy can be tried (Coulet *et al.*, 1991).

5.1.3. Aims.

The aims of this chapter were:-

- improve the working electrode ink in terms of catalytic properties and stability by changing its formulation;
- improve the membrane structure of the sensor to prevent fouling and interferences by developing the cellulose acetate and polyurethane coatings empirically;
- apply mass fabrication technologies (screen printing and Cavro solution deposition) to the whole production of the sensor;
- change the design of the sensor to a miniature device applicable to invasive monitoring and to one which allows characterisation using flow injection analysis;
- assess the behaviour of the sensor in buffer, plasma and blood to simulate an *in vivo* situation.

To produce the most sensitive and stable amperometric enzyme electrode for L-lactate, the fabrication of the sensor needs to be improved. Based on the work carried out in Chapters 2 and 3, it was the intention to improve the catalytic properties and the stability of the screen-printing ink by altering the rhodinised carbon and lactate oxidase content. The way in which the ink was formulated would also need investigation. Since the sensor was intended for operation in whole blood, a strategy for reducing the fouling and interferences was needed, and the work carried out in Chapter 4 was used as a basis. As discussed earlier, the sensor needs to be characterised, both in buffer and biological solutions leading to the possibility of *in vivo* assessment.

5.2. EXPERIMENTAL.

5.2.1. General Reagents.

General reagents were used as described in Section 2.2.1.

5.2.2. Rhodinised Carbon Inks.

Rhodium on carbon (metallised carbon, 5% w/w metal on graphite) was purchased as powders from Aldrich Chemical Company (Gillingham, Dorset) and Avocado Research Chemicals (Heysham, Lancashire). The rhodinised carbon was crushed using a pestle and mortar to produce a finer powder and precautions were taken to avoid contact or inhalation of metallised carbons. A screen printable ink was made by dispersing the metallised carbon powder in a solution of hydroxyethyl cellulose (2% w/v in buffer) or cellulose acetate (4% w/v in a 1:1 v/v acetone to cyclohexanone solution) with a small amount of finely milled carbon powder (T15 graphite from Lonza G and T Limited, Sins, Switzerland) to make a smooth paste. The ink consistency was optimised for both printing ability and electrochemical response, by adding different proportions of reagents. The inks were printed onto the eight array electrodes using the water-resistant working electrode screen with the DEK 245 screen printer as previously described (Section 2.2.4.). The electrodes were dried at room temperature for 2 hours and stored in the dark.

5.2.3. Lactate Oxidase Inks.

Two types of enzyme inks were formed: a homogeneous ink combining the lactate oxidase with the rhodinised carbon ink which was printed directly on the carbon overlay pad, and lactate oxidase combined with a carbon graphite ink which was printed on top of the rhodinised carbon surface (referred to as layered ink). The enzyme inks were adapted from work reported by Hart *et al.* (1996) where lactate oxidase (LOD) and carbon powders (rhodinised carbon from Avocado Research Chemicals, Lancashire, and/or T15 graphite from Lonza G and T Limited, Sins, Switzerland) were dispersed in hydroxyethyl cellulose solution (HEC, 2% w/v in buffer) in a weight ratio 1:10:18 (LOD:C:HEC). The inks were mixed on a rotational stirrer for 30 to 60 minutes before printing commenced using the water-resistant working electrode screen as previously described (Section 2.2.4.). The lactate oxidase electrodes were dried at room temperature for approximately 1 hr and stored

with silica gel at 4°C. A retaining membrane of cellulose acetate (2% w/v in acetone) was applied over the electrode surface by dipping the electrode into the cellulose acetate solution and allowing it to dry vertically for at least one hour.

5.2.4. Screen Printing Miniature Sensors and Three Electrode Sensor Arrays.

A high performance multi-purpose precision screen printer DEK 245 (DEK, Weymouth) was used to manufacture planar arrays of three miniature electrodes in several stages. Transparency film for plain paper copiers (PP 2500, 3M, France) was used as a substrate onto which a sequence of patterns was printed (see Section 2.2.4.). Although the pattern was slightly different, the screens were fashioned as described earlier in Section 2.2.4. and the same inks were used. A quick drying screen printing ink with high resistance to water penetration was used as insulation shroud (Light Green, Glanz Jet HG 40/NT-Neu, Wiederhold Siebdruckfarben, Coates Brothers GmbH, Nurnberg, Germany) and dried in air for 30 minutes. A rhodinised carbon with lactate oxidase ink, as described above (Section 5.2.3.) was printed on the working surface.

The three electrode planar array for use in a flow cell was printed onto a poly(vinyl) chloride substrate (PVC, Genotherm, Sericol, Surrey, UK) employing a multipurpose precision screen printer DEK 247 (DEK, Weymouth). Similar screens and inks were used, the quick drying screen printing ink with high resistance to water penetration was used as insulation shroud (Light Green, Glanz Jet HG 40/NT-Neu, Wiederhold Siebdruckfarben, Coates Brothers GmbH, Nurnberg, Germany) and the same drying procedures were followed as in Section 2.2.4.

5.2.5. Membrane Solutions.

Cellulose acetate (acetyl content approximately 40%) was made into solutions in acetone (99% purity) or in a acetone-cyclohexanone mixture (1:1 by volume). Polyurethane (Pellethane, 2363-80AE from Dr. D. Pfeiffer, Bio Sensor Technologie, Berlin, Germany) was solubilised in 98% tetrahydrofuran (99%, without stabiliser) and 2% N,N'-dimethyl formamide. Concentrations referred to in the text are percentage weight for volume.

5.2.6. Dip Coating Method of Membrane Application.

The sensor was immersed in an appropriate concentration of relevant membrane solution and withdrawn almost immediately. The drips of solution were removed either on the edge of the vessel or on tissue paper and the sensor was left to dry vertically, for at least an hour.

5.2.7. Pipetting Membrane Solutions.

An appropriate volume of membrane solution (2 – 3 μl for single electrodes) was drawn into a pipette (Pipetteman by Gilson, supplied by Anachem Bioscience, Anachem Limited, Luton, Bedfordshire) and then applied evenly to the electrode surface. The electrode was left to dry in a horizontal position to prevent the spread of solution.

5.2.8. Cavro Solution Deposition Technique.

Membrane solutions were also deposited by a programmable, precision liquid handling instrument designed by Cavro Scientific Instruments Incorporated, Sunny Vale, California (adjusted and supplied by Biodot Incorporated, Diddington, UK). The Cavro instrument (Cavro) is a stepper motor driven syringe pump which is controlled by an external microprocessor. It works as a slave unit, automating pipetting and dispensing functions, illustrated in Figure 5.1.

A pattern and repeats are programmed into the hand-held microprocessor along with details of flow rate and volume. A two-way syringe pump draws up the sample fluid and dispenses it through a needle, attached to a moveable arm. The arm moves by means of compressed air (approximately 1 bar).

5.2.9. Batch Measurements.

Hydrogen peroxide, L-lactic acid and ascorbic acid calibrations were carried out in the following manner. A three electrode system comprising a working electrode, saturated Calomel reference (SCE, Russell, Auchtermuchty, Fife) and platinum wire auxiliary (0.5 mm, BDH Limited, Poole, Dorset) was used in conjunction with an Autolab (EcoChemie BV, Utrecht, The Netherlands) in amperometric mode (GPES3 software). The electrodes were immersed in a 10 ml stirred buffer solution and an operating potential of +400 mV (SCE) was applied. Once a steady background current was achieved, a known volume of sample

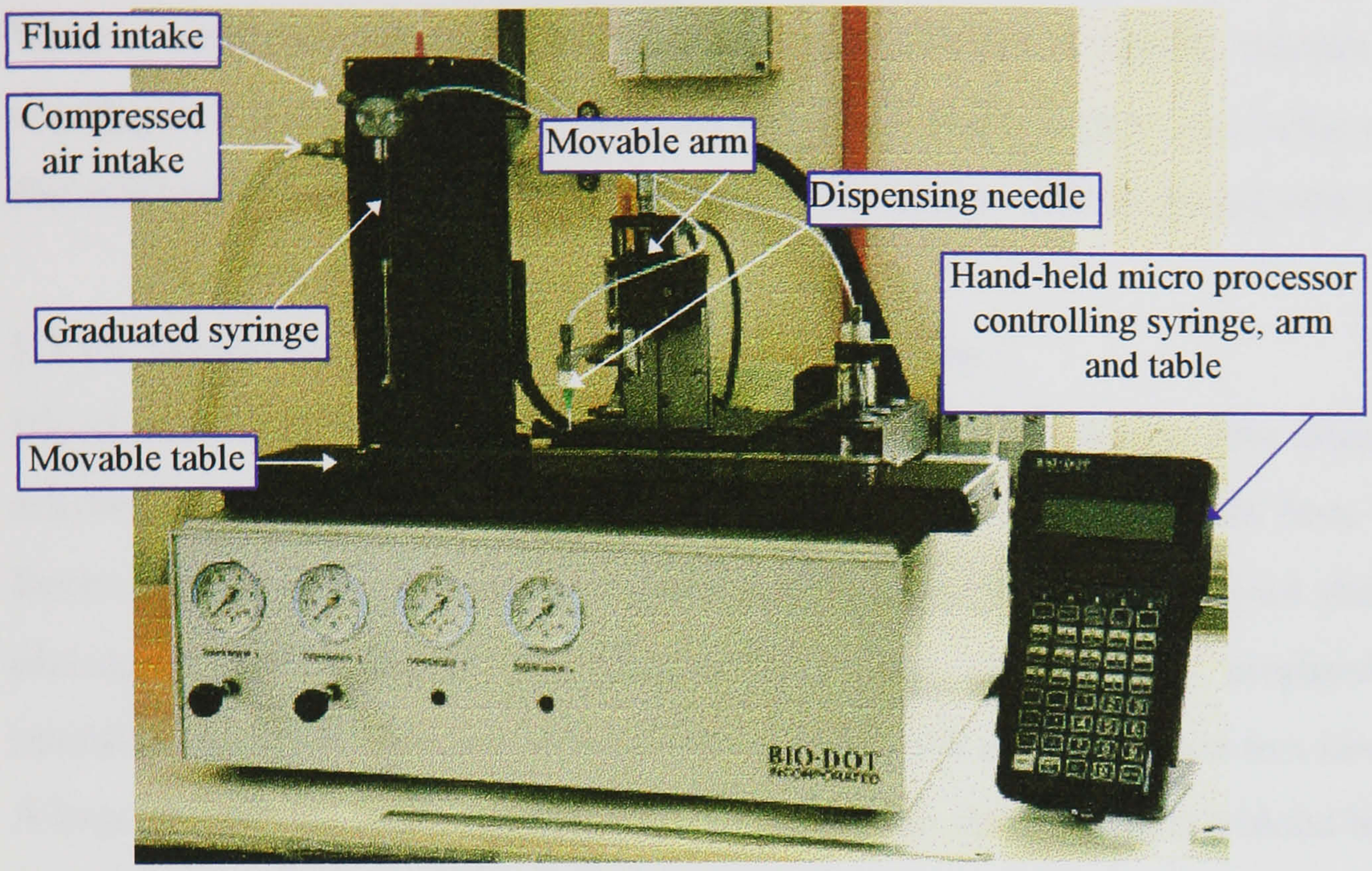


Figure 5.1. Annotated image of a Cavro printer.

solution (buffer containing either 10 M hydrogen peroxide, 0.2 M lithium L-lactate or 0.5 M sodium salt of L-ascorbic acid) was injected into the cell, away from the working electrode. When a steady current was again reached, another aliquot of sample solution was added, resulting in a step-like response. Once the calibration was complete, the buffer solution was discarded and the electrodes rinsed with clean buffer before refilling the cell with 10 ml buffer.

5.2.10. Whole Human Blood and Plasma Collection.

Blood was drawn from consenting humans with a minimum tunicae into tubes containing ethylenediaminotetraacetic acid (EDTA, 3 or 5 ml purple topped vials from Vacutainer, Becton Dickinson Company). The tubes were inverted several times (not shaken) before placing at on ice before use. Plasma from these samples was prepared by gentle centrifugation (6500 rpm for 10 min) and removing the upper solution to a new container. A large volume of standard whole human plasma was obtained from a blood bank (Herlev Hospital, Copenhagen, Denmark) which had been supplied by a donor. Health and safety protocols for the handling of blood were taken into consideration throughout all experiments using blood and its products.

5.2.11. Commercial Diagnostic L-lactate Determination.

Immediately after blood was withdrawn, it was placed for analysis in a Ciba Corning 860 whole blood analyser with a lactate attachment (Chiron Diagnostics part of Ciba Corning, Essex, England). A mechanism automatically identified the sampling device and drew the precise volume required. This was taken into the flow stream and passed a series of sensors, including an amperometric lactate array. A display and print-out was obtained of blood pH, oxygen tension, carbon dioxide tension, sodium, potassium, calcium and chloride ions, and haematocrit, glucose and lactate content.

Alternatively, a quantitative enzymic determination of L-lactate present in the plasma fraction of whole blood was carried out using a diagnostic kit (Lactate reagent 735-10, Sigma Diagnostic, Sigma-Aldrich Company Limited, Poole, Dorset). Blood was centrifuged (1650 g, 8 min) and the upper, plasma fraction was used for L-lactate determination. Lactate reagent (1 ml) and sample (7.5 μl) were mixed in cuvettes (1 cm path length), incubated at room temperature for approximately 10 minutes and then the absorbance at 540 nm was

recorded (uv-visible spectrophotometer, Camspec M350). The absorbance of the plasma fraction was correlated to the L-lactate concentration using a calibration plot previously obtained from solutions of known L-lactate concentration.

5.2.12. Flow Injection Analysis.

For early plasma and blood analysis, a flow injection system similar to Figure 5.2. was set up. These analysis were undertaken in a clinical laboratory at Herlev Hospital, Copenhagen, Denmark with health and safety procedures followed throughout. The flow cell was connected to a six port valve (FIAstar 5102-002 injector 'v-200') controlled by a FIAstar 5020 Analyser (supplied by Tecator AB, Hoganas, Sweden). The sample solution was peristaltically pumped into a loop with an approximate volume of 100 μl, whilst buffer was continually pumped by a syringe infusion pump (Harvard Apparatus) through the valve and cell at a rate of 0.5 $ml.min^{-1}$. A sample entered the cell every 198s as a discrete plug. The cell was connected to a miniature potentiostat controlled by Windows based software (designed and built by Mr. C. Bessant, Cranfield Biotechnology Centre, Cranfield University, Bedfordshire) driven by a lap-top computer (Viglen Dossier, 486 SD). The computer program continually applied a potential of +400 mV (Ag/AgCl) and recorded the current output, enabling the data to be stored and analysed at a later date.

A flow system as shown in Figure 5.2. incorporating a flow cell with an approximate volume of 280 μl was also used. The electrodes were linked to an Autolab (GPES3 software, EcoChemie BV, Utrecht, Holland) in chronoamperometry mode with an operating potential was +350 mV (Ag/AgCl). The flow cell was connected to a flow injection system comprising of a software driven key pad control device (Gilson, France) linked to two diluter syringe pumps (capacity of 5 ml, Model 401C, Gilson, France) and an injector valve actuator (6 ports, Model 817, Gilson, France). The flow rate, injection frequency and number of injections are set via the control pad; the conditions for L-lactate analysis in buffer, plasma and blood were 0.75 $ml.min^{-1}$ flow rate, with 1 ml buffer between each injection which had an approximate volume of 200 μl. Phosphate buffered saline (0.1 M phosphate, 0.15 M sodium chloride, pH 7.2) was used in all experiments.

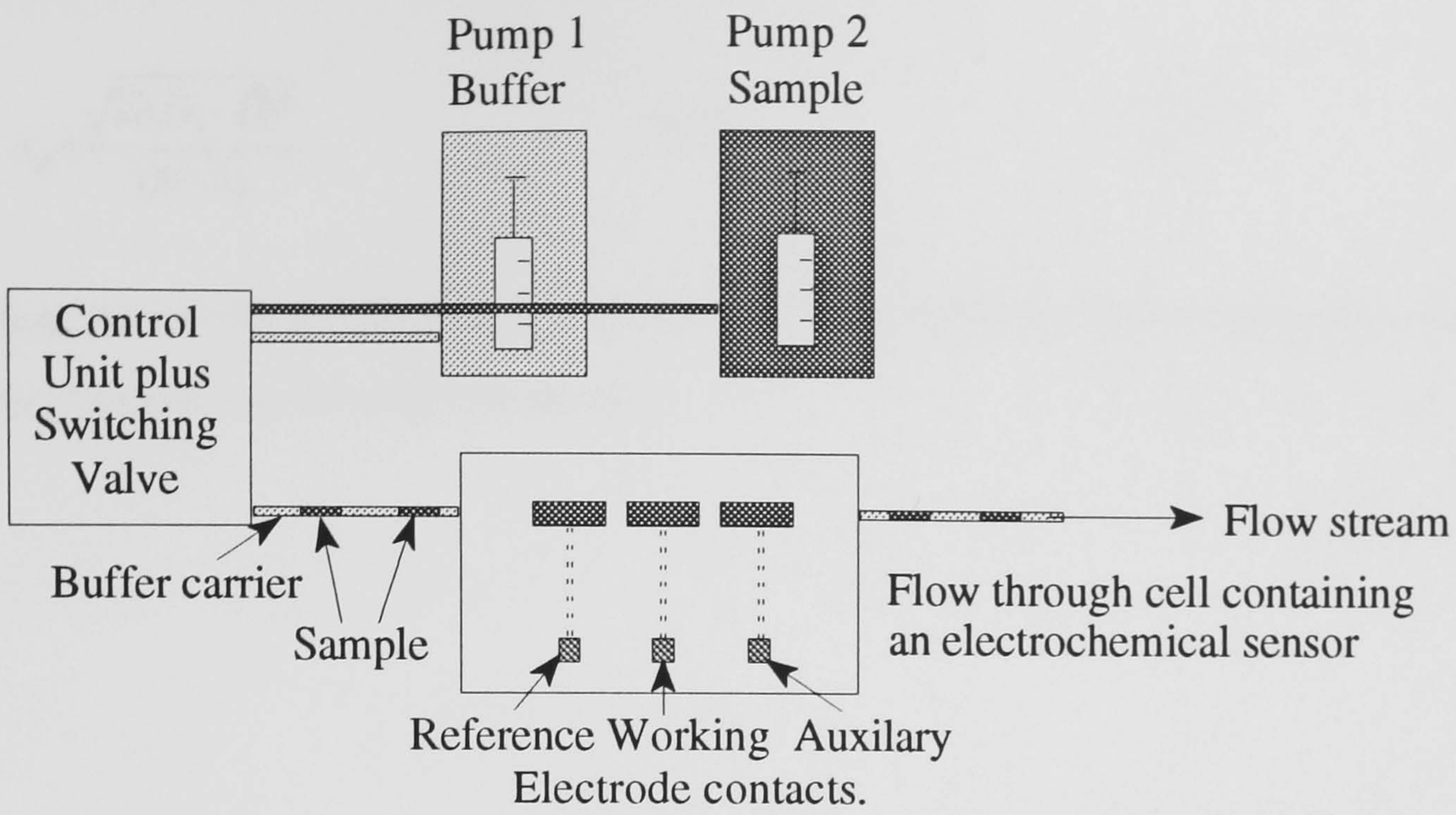


Figure 5.2. Schematic diagram of flow injection analysis apparatus.

5.2.13. Measurement and Presentation of Data.

The measurement and presentation of data were performed as described in Section 2.2.20.

5.2.14. Statistical Analysis.

Two tests of significance were carried out, the *F* test and the Student *t* test. *F* is defined in terms of the variances of the two methods:

$$F=\frac{s_1^2}{s_2^2}$$

where s^2, the variance, is the square of the standard deviation (s) and where the degrees of freedom is defined as the number of tests minus one, for each case. The calculated *F* value was then compared to tabulated F values at the 99.9% confidence level.

The Student *t* test with multiple samples was carried out. This tested the sensor against an accepted method by analysing several different samples. The difference between each of the paired measurements on each sample was computed. The average difference $\bar{D}$ was calculated and the individual standard deviations of each from $\bar{D}$ are used to compute a standard deviation, s_d. The *t* value was then calculated from:

$$s_d = \frac{\sqrt{\Sigma(D_i - \bar{D})^2}}{(N-1)} \qquad \text{and} \qquad t = \frac{\bar{D}}{S_d}\sqrt{N}$$

where D_i is the individual difference between the two methods for each sample. The *t* value was then compared with tabulated values.

5.3. RESULTS.

5.3.1. Ink Improvement.

The composition of the rhodinised-carbon ink was assessed with respect to the catalytic ability and ease of printing. Several different rhodinised-carbon (5% rhodium on carbon by weight) inks were printed onto base electrodes and coated with a cellulose acetate membrane (4% w/v in acetone) and their response to hydrogen peroxide was evaluated. The inks are described and the results are shown in Figure 5.3.

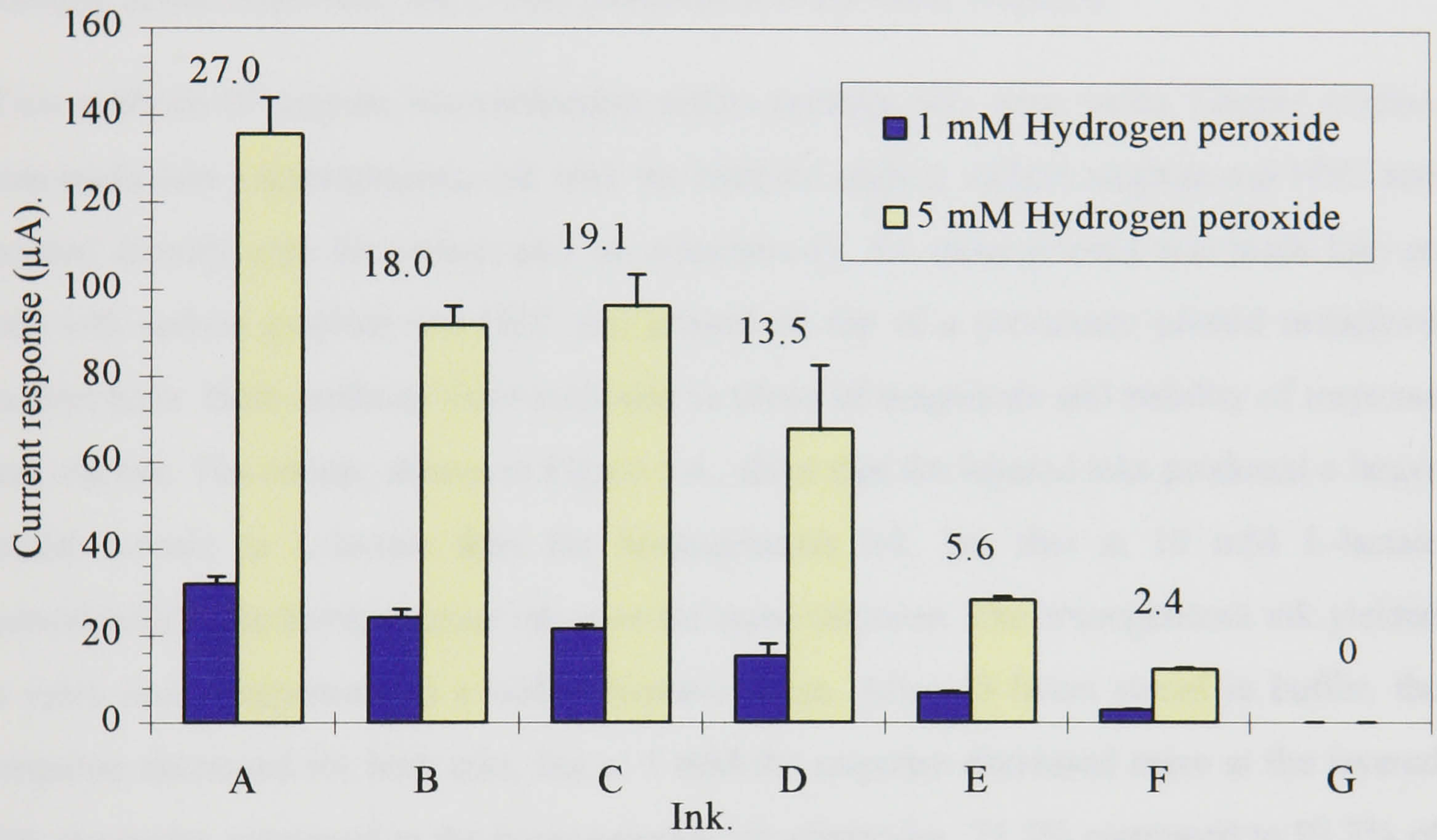


Figure 5.3. Response to hydrogen peroxide of a variety of rhodinised carbon ink formulations at +400 mV (SCE). Triplicate measurements on three electrodes, number represents the slope of the response (from 0 to 5 mM hydrogen peroxide). Ink formulation listed below:

A Avocado rhodinised carbon with HEC in a ratio of 1:2

B Avocado rhodinised carbon with carbon graphite and HEC in a ratio of 1:1:3

C Avocado rhodinised carbon with carbon graphite and HEC in a ratio of 4:1:9

D Avocado rhodinised carbon with cellulose acetate in a ratio of 1:1

E Aldrich rhodinised carbon with HEC in a ratio of 1:2

F Aldrich rhodinised carbon with carbon graphite and HEC in a ratio of 1:1:3

G Bare carbon overlay pad (Electrodag 423 SS)

The rhodinised carbon from Avocado performed the best in terms of printing ease and signal to hydrogen peroxide. The hydroxyethyl cellulose formed a more stable environment and printable ink for rhodinised carbon (comparing D and A) than cellulose acetate. As expected, by adding more carbon, the catalytic effect of the rhodium decreased (compare electrodes A, B and C) but carbon was required to allow the ink to flow smoothly across the screen without excessive drying during the printing process. The inks which were easiest to print were B and C. The values of the slopes of the responses to hydrogen peroxide show that ink C is more sensitive than B and from looking at the responses, ink C also produces a more linear response.

Two methods of enzyme immobilisation within printing inks were tested. Lactate oxidase was made into a homogeneous ink with the catalytic carbon, carbon graphite and HEC and printed directly onto the carbon pad, or, alternatively, the same amount was made into an ink with carbon graphite and HEC and printed on top of a previously printed metallised carbon layer. Both methods were evaluated in terms of magnitude and stability of response to L-lactate. The results, shown in Figure 5.4., show that the layered inks produced a larger initial current to L-lactate than the homogeneous ink, but that at 10 mM L-lactate concentration, the homogeneous ink gave the same response. The homogeneous ink yielded a more linear response and a wider dynamic range. After 18 hours stored in buffer, the response decreased for both inks, but at 5 mM the response decreased more at the layered ink electrodes compared to the homogeneous ink electrodes: 71.3% compared to 92.2% of original signal, respectively. The amount of lactate oxidase printed onto each electrode using the water based screen for the eight electrode design, was weighed and calculated to be approximately 1.8 U of enzyme, relating to 0.129 $U.mm^{-2}$.

The proportion of lactate oxidase and the way it was added to the catalytic printing ink was evaluated. Inks were made with HEC (180 mg, 2% w/v in buffer), rhodinised carbon (80 mg) and carbon graphite (20 mg) and then lactate oxidase was added in varying amounts from 1 to 20 mg. Alternative methods were to dissolve the lactate oxidase in the HEC before adding the carbons, or to add the lactate oxidase, in a small amount of buffer, to the carbons and allow the water to evaporate (at 4°C for 24 hours) before adding the HEC. Figure 5.5. shows the L-lactate calibration curves of electrodes printed with different inks.

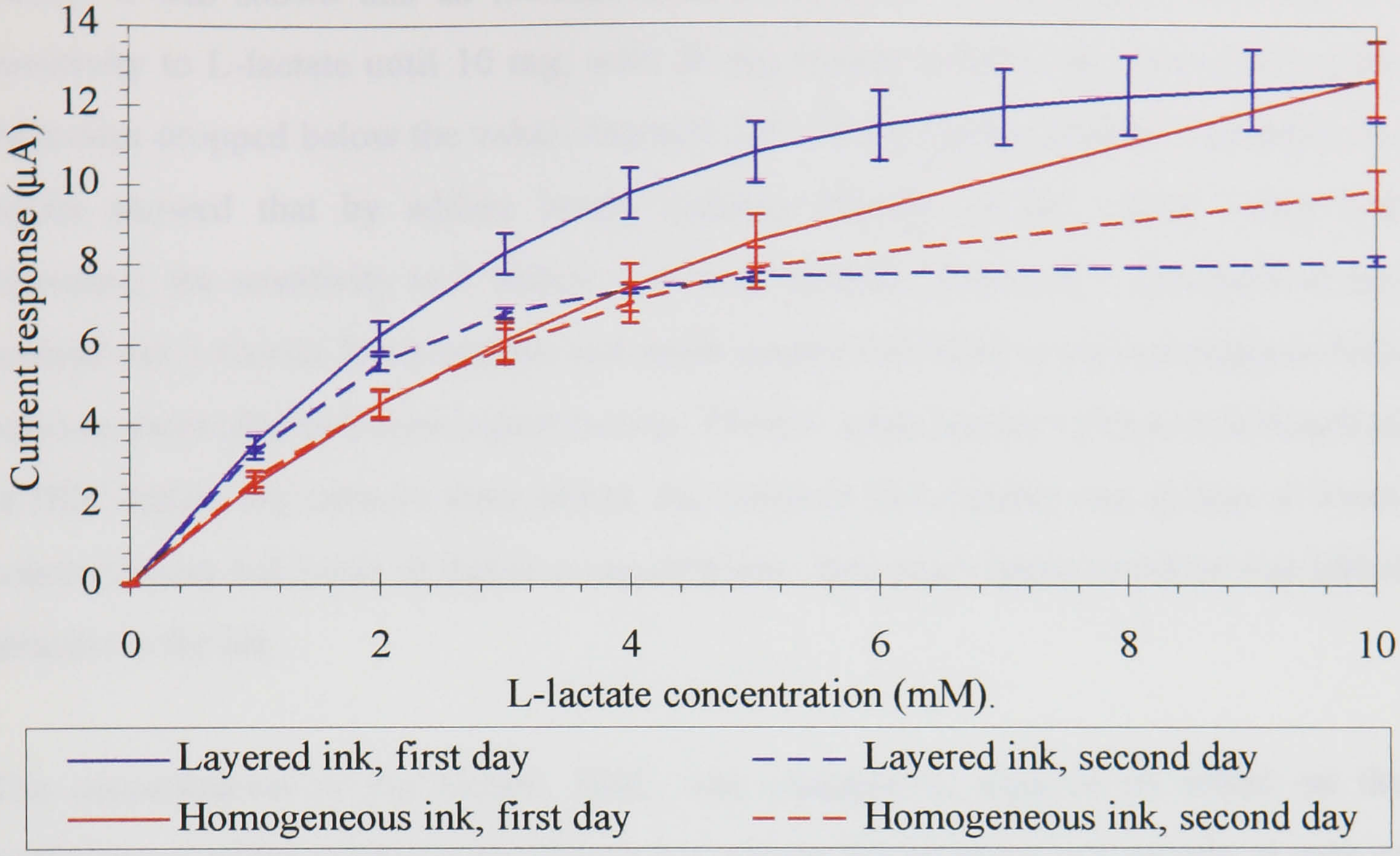


Figure 5.4. Comparison of two methods of lactate oxidase ink formulation. Current response to lactate at +400 mV (SCE) over two days. Triplicate tests on three electrodes.

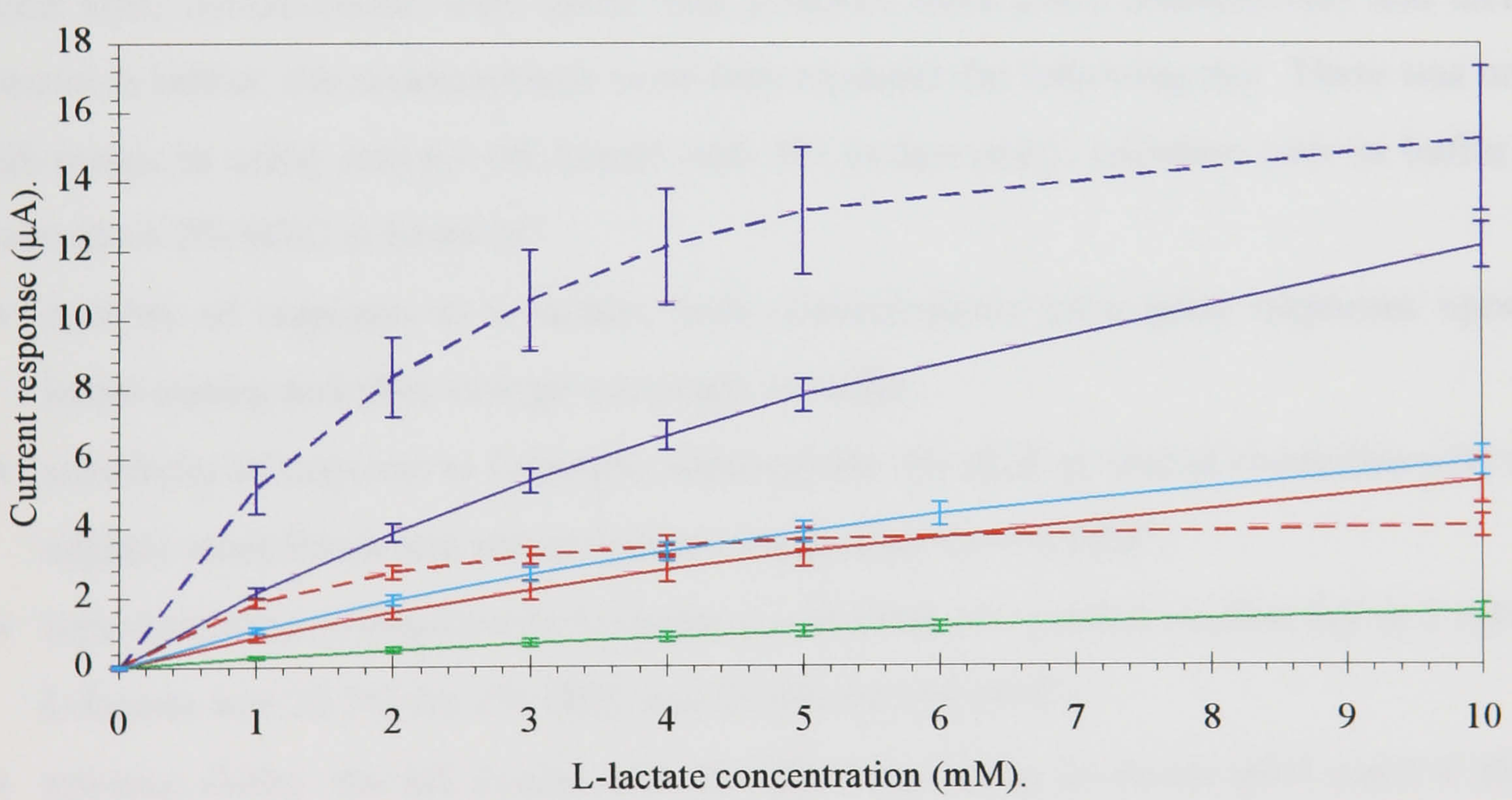


Figure 5.5. Current response to L-lactate of enzyme electrodes with different amounts of lactate oxidase, at +400 mV (SCE). Three electrodes tested in triplicate on two days, apart from the 10 mg LOD electrodes which are means and standard errors of three electrodes tested on one day only.

Firstly, it was shown that an increase in lactate oxidase concentration increased the sensitivity to L-lactate until 10 mg; with 20 mg lactate oxidase, the sensitivity of the electrodes dropped below the value obtained with 10 mg lactate oxidase. Secondly, the results showed that by adding lactate oxidase directly to the carbon before ink formation, the sensitivity to L-lactate was much greater. However, a drawback of this method was a shorter linear portion and much greater deviation in current response both between electrodes and upon repeat testing. Thirdly, when lactate oxidase was dissolved in HEC before the carbons were added, the response to L-lactate was greater at lower concentrations but lower at higher concentrations, than when lactate oxidase was added directly to the ink.

The concentration of the binder, HEC, was changed to observe its effect on the performance of the catalytic ink. Figure 5.6. shows the response to L-lactate at sensors fabricated with either 2% or 4% HEC over a two day testing regime. Two sensors of each HEC concentration were tested with L-lactate three times consecutively and then stored in buffer; the measurements were then repeated the following day. There was no advantage in using enzyme ink bound with 4% hydroxyethyl cellulose (w/v in buffer) instead of 2% HEC in terms of:

- stability of response to L-lactate; both concentrations gave good responses upon initial testing and after storage overnight in buffer;
- sensitivity of response to L-lactate; although the 2% HEC produced electrodes with a slightly more linear and wider dynamic range than the 4% HEC;
- reproducibility of response to L-lactate (coefficient of variation on first day at 2 mM L-lactate was 22.2% for 2% HEC and 25.0% for 4% HEC);
- printing ability; the ink bound with 2% HEC was easier to screen print since it did not dry as quickly as the ink bound with 4% HEC.

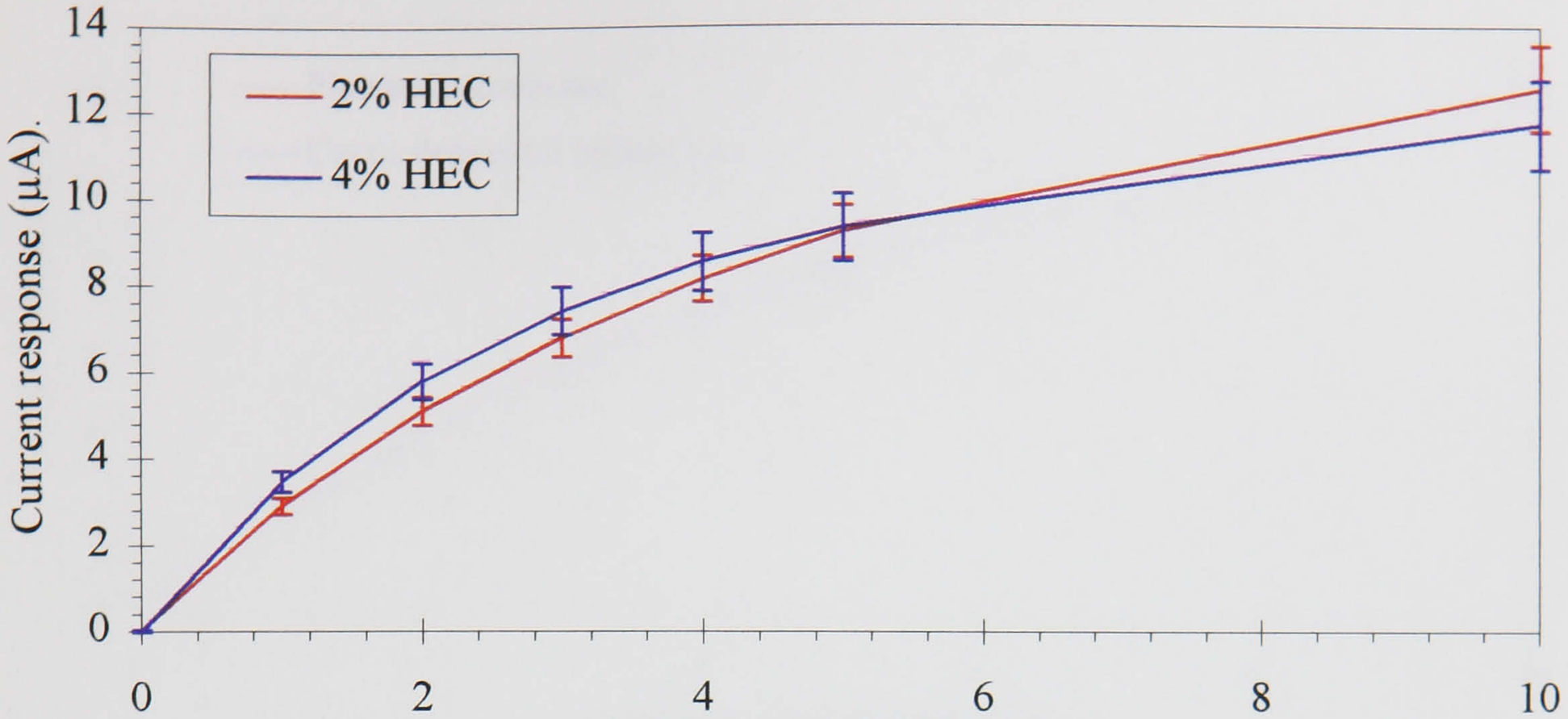


Figure 5.6. L-lactate calibration of sensors fabricated with inks made with either 2% or 4% hydroxyethyl cellulose. Sensors coated with 2% cellulose acetate (3 µl) and tested at +400 mV (SCE). Test performed over two days, two sensors of each HEC concentration consecutively tested in triplicate.

5.3.2. Membrane Deposition.

The possibility of automated membrane deposition was explored. Electrodes coated with membranes applied by the Cavro device were compared with electrodes coated with membranes applied by pipetting by hand. The current response to 0 - 10 mM L-lactate was recorded for seven electrodes from each application batch. The results, illustrated in Figure 5.7., showed that the response to L-lactate and standard errors were very similar, although the electrode coated with Cavro deposited membrane were slightly more precise at detecting L-lactate than electrodes with hand-pipetted membranes. The coefficient of variation of current response to 2 mM L-lactate was 28.7% and 23.1% for pipetted and Cavro deposited membranes, respectively.

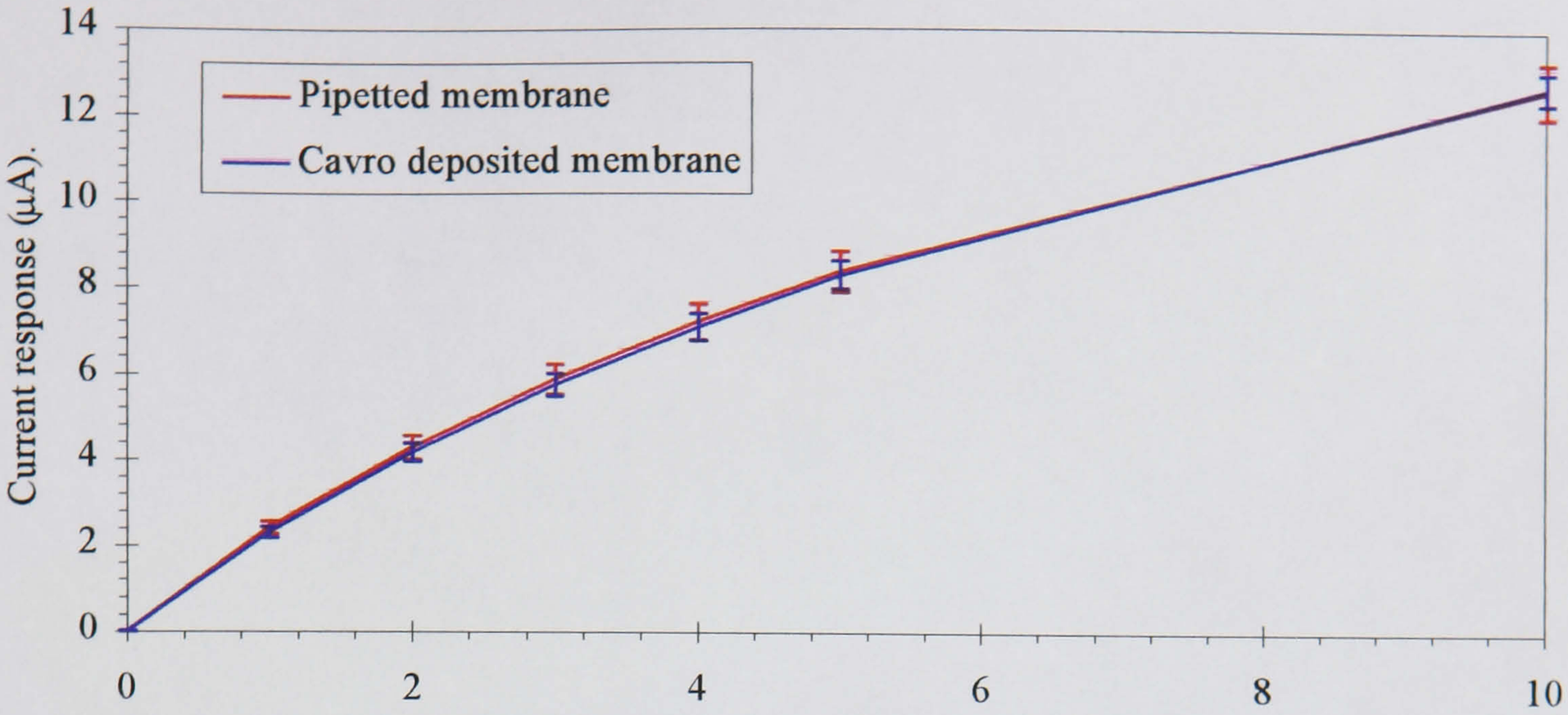


Figure 5.7. Response to lactate by lactate oxidase enzyme electrodes with cellulose acetate membrane applied in two different manners. Seven electrodes tested in triplicate with the potential held at +400 mV (SCE).

5.3.3. Sensor Design.

Screen printed electrodes were designed for use in a flow injection system (Figure 5.8.) or indwelling in a catheter or needle (Figure 5.9.). Three electrodes, one alongside the other, were printed onto a card with allocation holes to align them in the flow cell. The flow of buffer and sample would pass from the reference to the auxiliary electrode. Each electrode had a surface area of 10 x 2 mm.

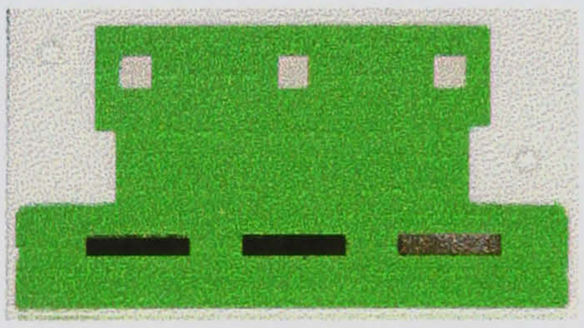
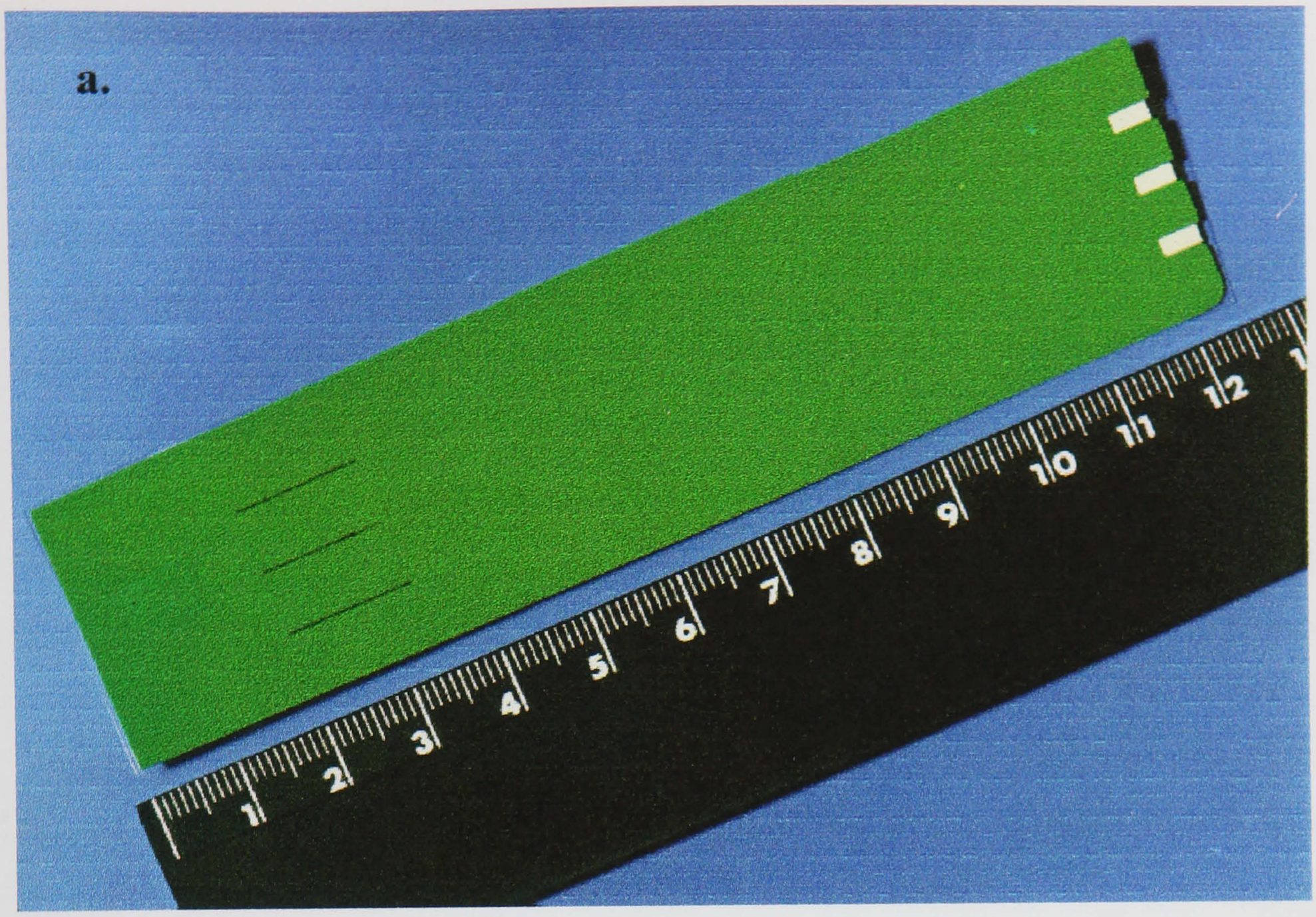


Figure 5.8. Three electrode sensor array for flow injection analysis. Green insulation shroud surrounds the contacts (top three squares) and the electrodes (bottom three oblongs). From left to right is the secondary electrode (carbon pad), working electrode (rhodinised carbon with lactate oxidase) and silver/silver chloride reference electrode.

Miniature electrodes for indwelling within a needle or catheter had a working surface of 0.5 x 15 mm and length of 105 mm.



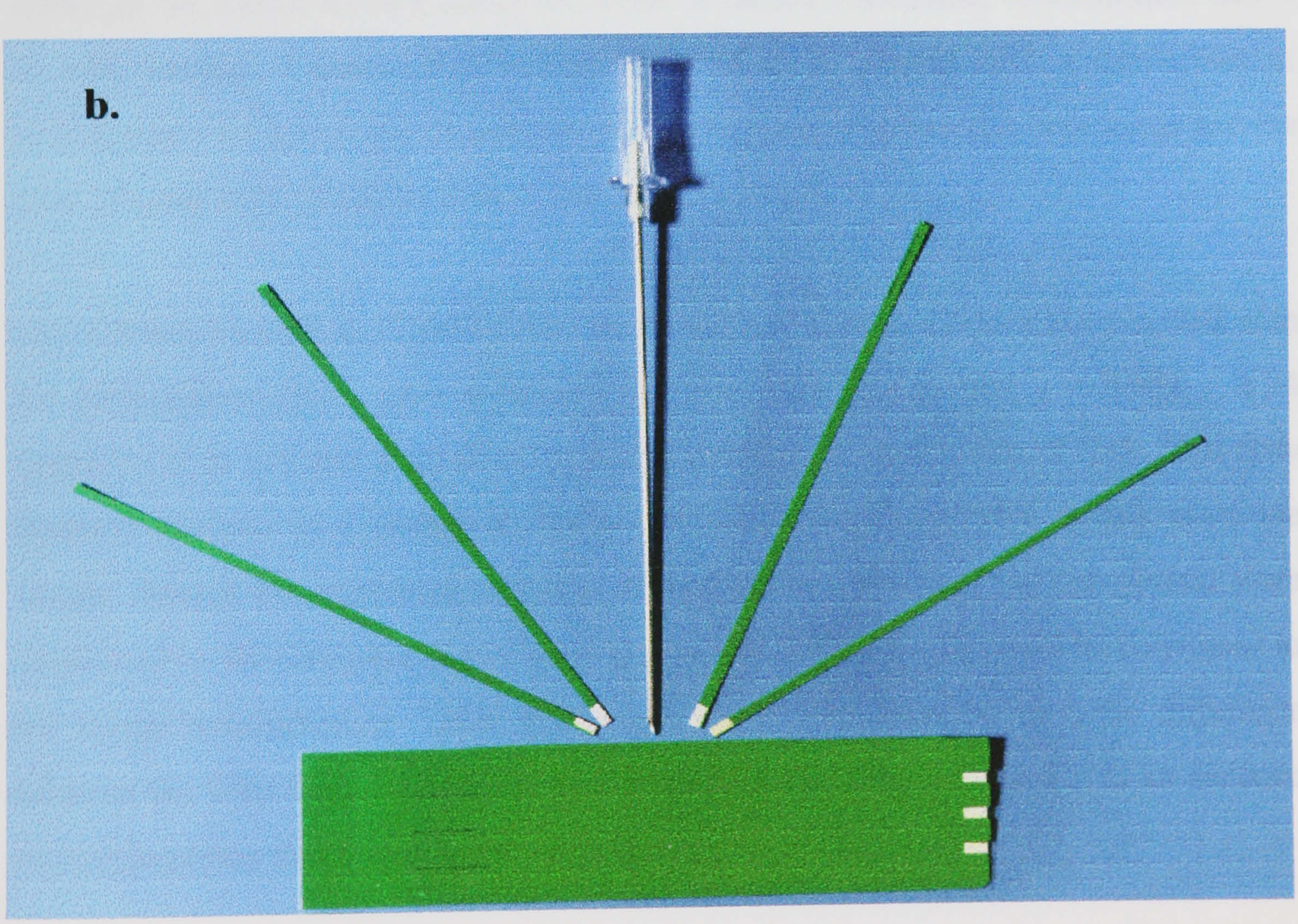


Figure 5.9. Pictures of miniature screen-printed electrodes in an array: a, three sliver screen-printed electrodes alongside a centimeter scale; b, three sensors at the bottom and four individual sensors with an 18-gauge catheter-needle in the center.

5.3.4. Initial Assessment of Lactate Oxidase Enzyme Electrodes in Plasma and Blood.

An appraisal of the function of polyurethane and cellulose acetate coated L-lactate enzyme electrodes in flow injection analysis (FIA) of buffer, plasma and blood was carried out. Three electrode sensor arrays for use in the FIA were dip coated in polyurethane (4% and 2% w/v in 98:2 tetrahydrofuran to dimethyl formamide) or cellulose acetate (2% w/v in acetone) and calibrated with L-lactate in buffer before testing with plasma or blood.

A sensor coated with 4% polyurethane was calibrated with L-lactate in buffer before being tested for long term stability in whole human plasma. A straight line was obtained with equation $y = 0.74x + 2.84$ ($r^2 = 0.998$, where y is in μA and x is in mM) for four 1, 2 and 10 mM L-lactate injections (coefficients of variation were 2.6%, 2.0% and 5.5%, respectively). Figure 5.10. shows a typical result of FIA with L-lactate and continuous plasma injections. The whole human plasma sample contained 2.16 mM lactate. Although the sensor did not detect this level of lactate (calculated concentration above 20 mM lactate) it produced a reproducible signal to plasma with a coefficient of variation of 9.81% over the 20 minutes it was analysed. The baseline was stable and the level of noise only increased from 0.066 μA to 0.18 μA.

On calibration with 1 to 10 mM L-lactate in the flow system, a sensor array coated with 2% polyurethane (w/v) gave a straight line plot with an equation $y = 0.68x + 2.24$ (where y is in μA and x is in mM) and a correlation coefficient of $r^2=1$. The flow injection analysis of L-lactate in buffer and whole human plasma at this electrode clearly showed the injections of L-lactate and when samples of spiked plasma were introduced to the electrode, a current response was gained which could be correlated to the lactate concentration. Table 5.1 shows the values obtained. A large difference between the Ciba Corning and the sensor reading was obtained but it was an additive current implying that it was due to interference.

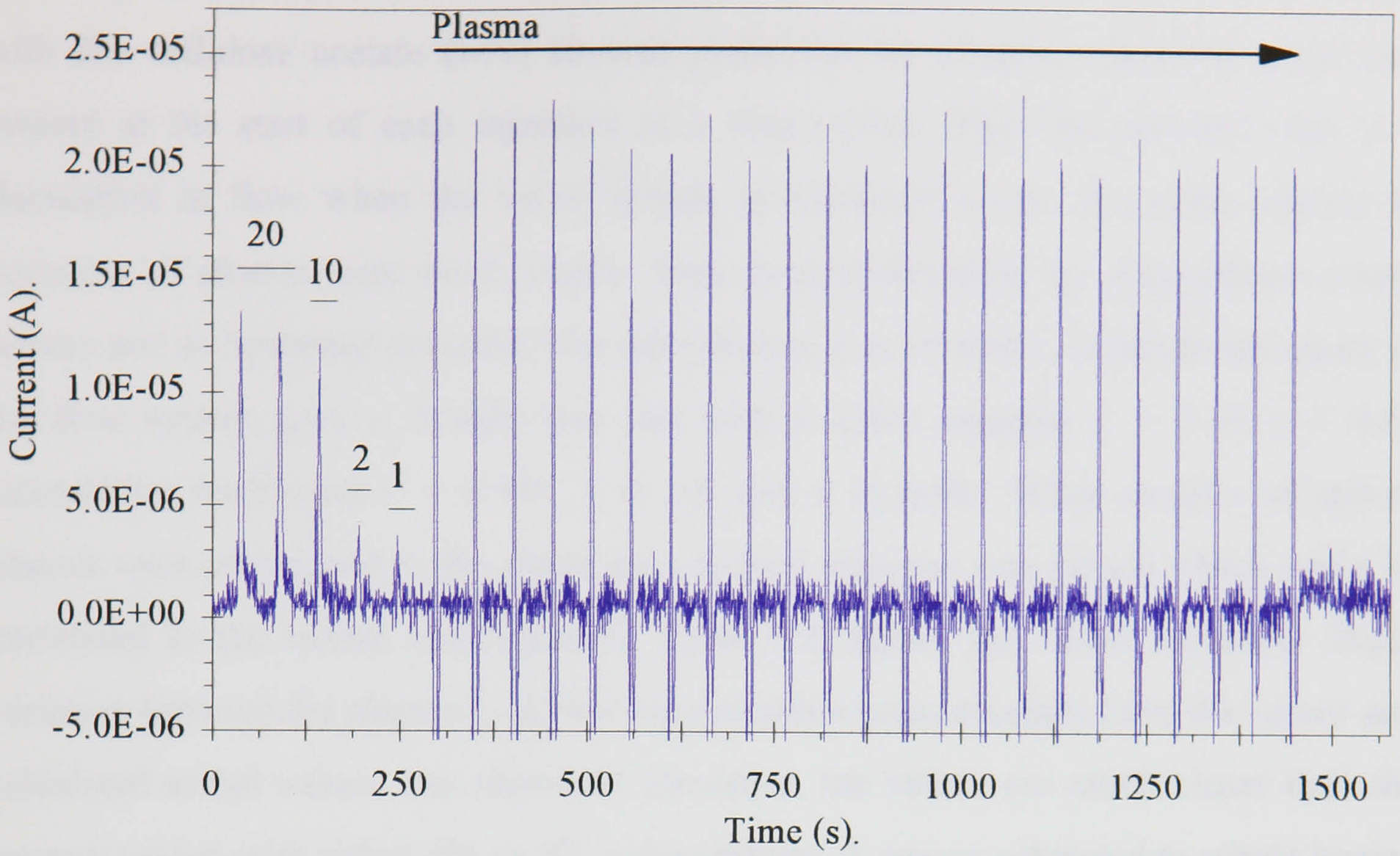


Figure 5.10. Flow injection analysis of lactate and plasma samples using a three electrode sensor with a modified working electrode, encased in polyurethane (4% w/v). Potential held at +400 mV (Ag/AgCl). Numbers represent lactate concentration of sample injection, 23 whole human plasma injections start after 1 mM lactate sample.

Table 5.1. Current response from flow injection analysis of whole human plasma samples with added lactate. Three electrode sensor with a modified working electrode, coated with polyurethane (2% w/v), two injections of each sample. Initial reading (*) measured by the Ciba Corning whole blood analyser.

Plasma lactate concentration	Response of sensor (μA ± S.D.).	Calculated concentration of lactate (mM ± S.D.).
2.16 mM*	15.65 ± 0.75	19.5 ± 1.6
4.16 mM	21.05 ± 0.35	27.5 ± 1.4
6.16 mM	21.80 ± 0.40	28.6 ± 1.5

Flow injection analysis of L-lactate and whole human plasma at a sensor array coated with 2% cellulose acetate (w/v) showed peaks for the samples, although noise was present at the start of each injection as a sharp peak. This was possibly due to a fluctuation in flow when the valve turned, or electrical noise. The peaks relating to injections of plasma were much smaller than those observed at the polyurethane coated sensors and no hysteresis occurred. The calibration to 1 to 10 mM L-lactate at this sensor in the flow system gave a straight line plot with a linear equation $y = 5.16 x + 4.09$ (correlation coefficient $r^2 = 0.998$, y in μA and x in mM). When samples of spiked plasma were introduced to the electrode a current response was gained which could be correlated to the lactate concentration. Table 5.2. shows the values obtained. Slight variation between the plasma L-lactate concentration measurements from the sensor and calculated actual values was observed. However, the values are much closer than the sensors coated with either 4% or 2% polyurethane. A sensor subjected to whole human plasma repeatedly, produced a repeatable response which only decreased by 1.9 % over 110 minutes. The sensor detected the lactate concentration of the plasma to be 3.89 mM and the Ciba Corning blood analyser gave a lactate concentration of 2.16 mM.

When blood was analysed by the L-lactate sensors coated with polyurethane, a very high response was attained. Figure 5.11 shows the FIA of L-lactate in buffer and blood at a sensor coated with 4% polyurethane. Although the analysis of blood did elicit peaks greater than those obtained by the highest L-lactate concentration in buffer, the responses were repeatable and blood spiked with L-lactate invoked a greater response than blood that was not spiked. It can be seen that after blood analysis, the current was slightly higher than and did not fully drop back to the original background current, and that the level of noise increased. Overall, the background current increased by 0.3 μA and the level of noise by 100% to 0.5 μA. The response to 10 mM L-lactate in buffer, after the injections of blood, was a consistent height and only decreased by 20.8% from the initial injections.

Table 5.2. Current response from a lactate sensor in the flow injection analysis of whole human plasma samples with added lactate. Three electrode sensor with a modified working electrode, coated with a cellulose acetate membrane (2% w/v). Readings marked with and asterisk (*) measured by the Ciba Corning whole blood analyser.

Plasma lactate concentration.	Response of sensor (μA ± SD).	Calculated lactate concentration (mM ± SD).
0.57 mM *	11.70 ± 1.13	1.48 ± 0.19
2.16 mM *	32.56 ± 0.97	5.52 ± 0.50
7.16 mM	49.37 ± 1.63	8.78 ± 0.81
12.16 mM	78.93 ± 0.92	14.51 ± 1.25

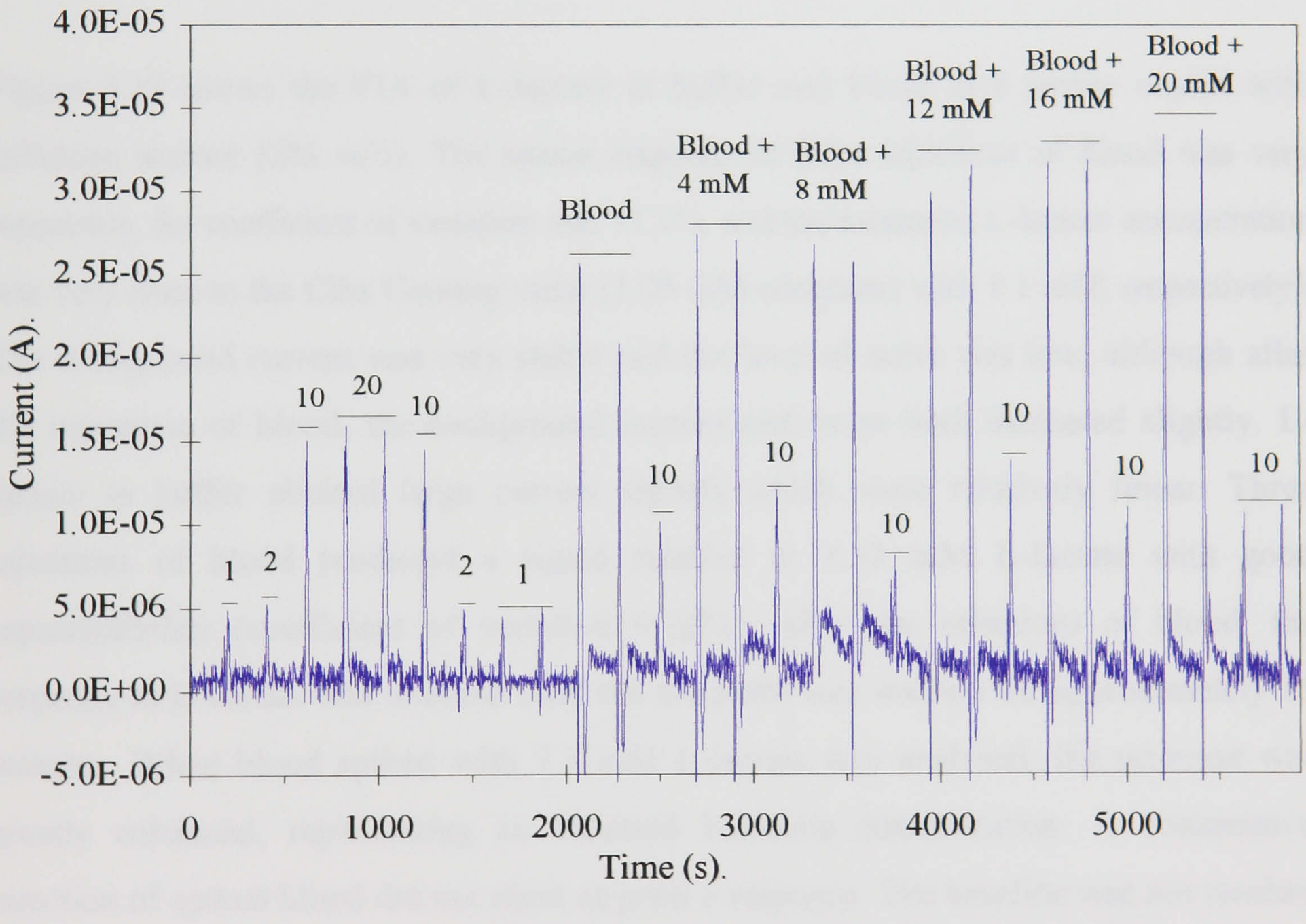


Figure 5.11. Flow injection analysis of lactate and blood samples using a three electrode sensor design with a modified working electrode, coated with a polyurethane membrane (4% w/v). Numbers represent lactate concentration of sample injection (in mM), spiked blood injections as illustrated. The Ciba Corning blood analyser gave a reading of 1.48 mM lactate concentration for the whole human blood.

When 2% polyurethane was used to coat the sensor, similar results were obtained. Figure 5.12. shows the FIA response of an L-lactate sensor coated with 2% polyurethane tested with L-lactate in buffer and blood. A residual current could be seen after the injections of 10 and 20 mM L-lactate in buffer. When blood was analysed, two peaks were recorded higher than the 20 mM L-lactate peak and showed more hysteresis. This meant that although the baseline current was not achieved before the next injection of blood, the sample elicited the same current response. Although the responses to L-lactate in buffer were not as large as those preceding the blood injections, the baseline was regained and the noise was only slightly increased. The final two responses to spiked blood were greater than the plain blood and produced uniform current signals but, again, the current did not drop to the baseline level before the next injection. Hysteresis was still present after 15 minutes in a stream of buffer.

Figure 5.13 shows the FIA of L-lactate in buffer and blood at a sensor coated with cellulose acetate (2% w/v). The sensor response to three injections of blood was very repeatable, the coefficient of variation was 11.1%, and the measured L-lactate concentration was very close to the Ciba Corning value (1.05 mM compared with 1.1 mM, respectively). The background current was very stable and the level of noise was low, although after the injections of blood, the background current and noise both increased slightly. L-lactate in buffer elicited large current signals which were relatively linear. Three injections of blood produced a signal relative to 1.52 mM L-lactate with good reproducibility (coefficient of variation 9.18%). After the injections of blood, the response to L-lactate was lowered until the electrode was washed for approximately 15 minutes. When blood spiked with 7.5 mM L-lactate was analysed, the response was greatly enhanced, representing an elevated L-lactate concentration. A consecutive injection of spiked blood did not elicit as great a response. The baseline was not reached after either injection and even after 9 minutes of washing with buffer, the baseline and noise were slightly elevated. A final injection of spiked blood showed that the electrode had recovered 87% of its activity although considerable hysteresis was again observed after the injection.

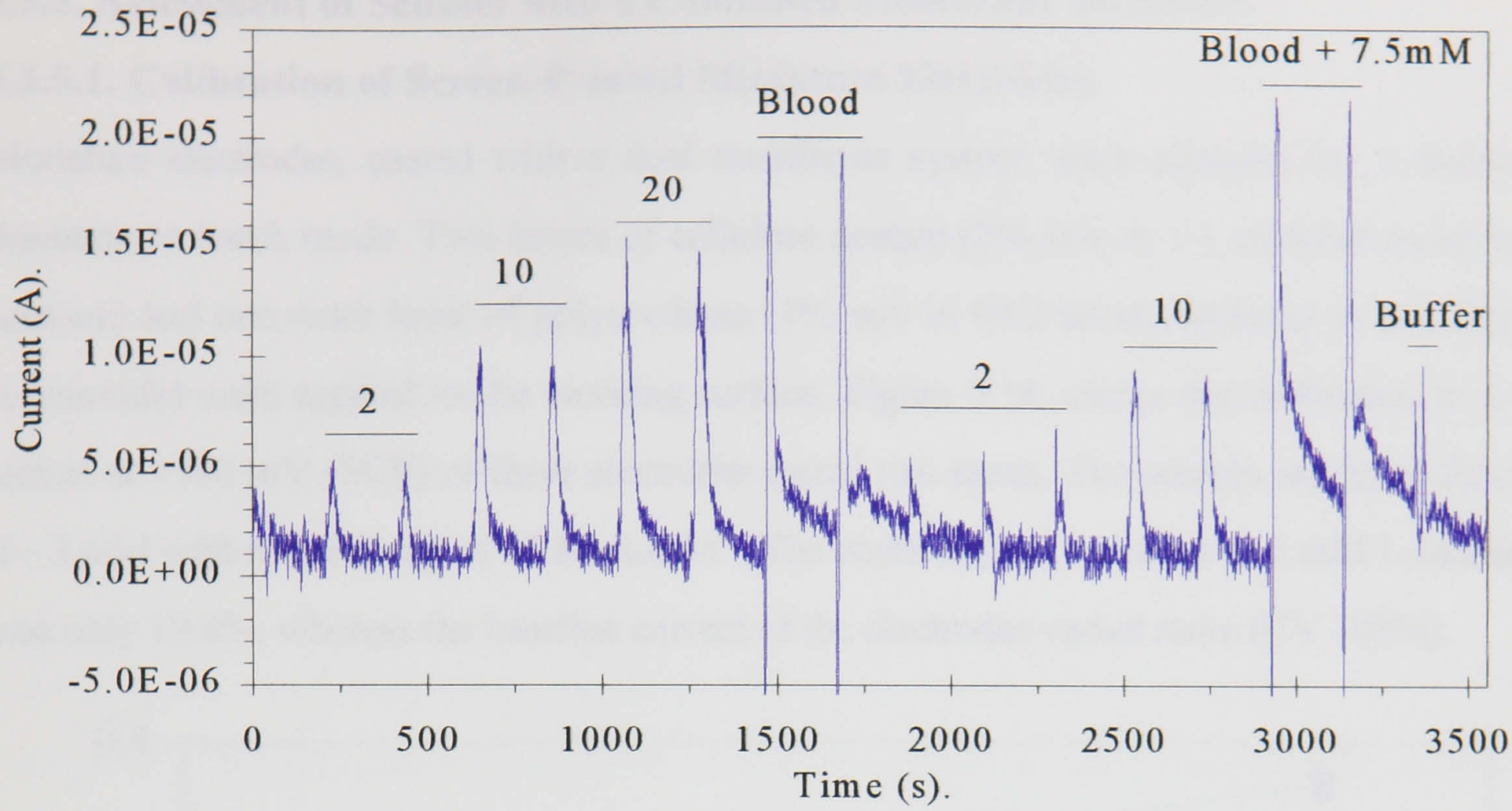


Figure 5.12. Flow injection analysis of lactate and blood samples using a three electrode sensor design with a modified working electrode, coated with a polyurethane membrane (2% w/v). Numbers represent lactate concentration of sample injection (in mM), whole human blood (lactate concentration of 1.1 mM) injections marked with spiked lactate concentration.

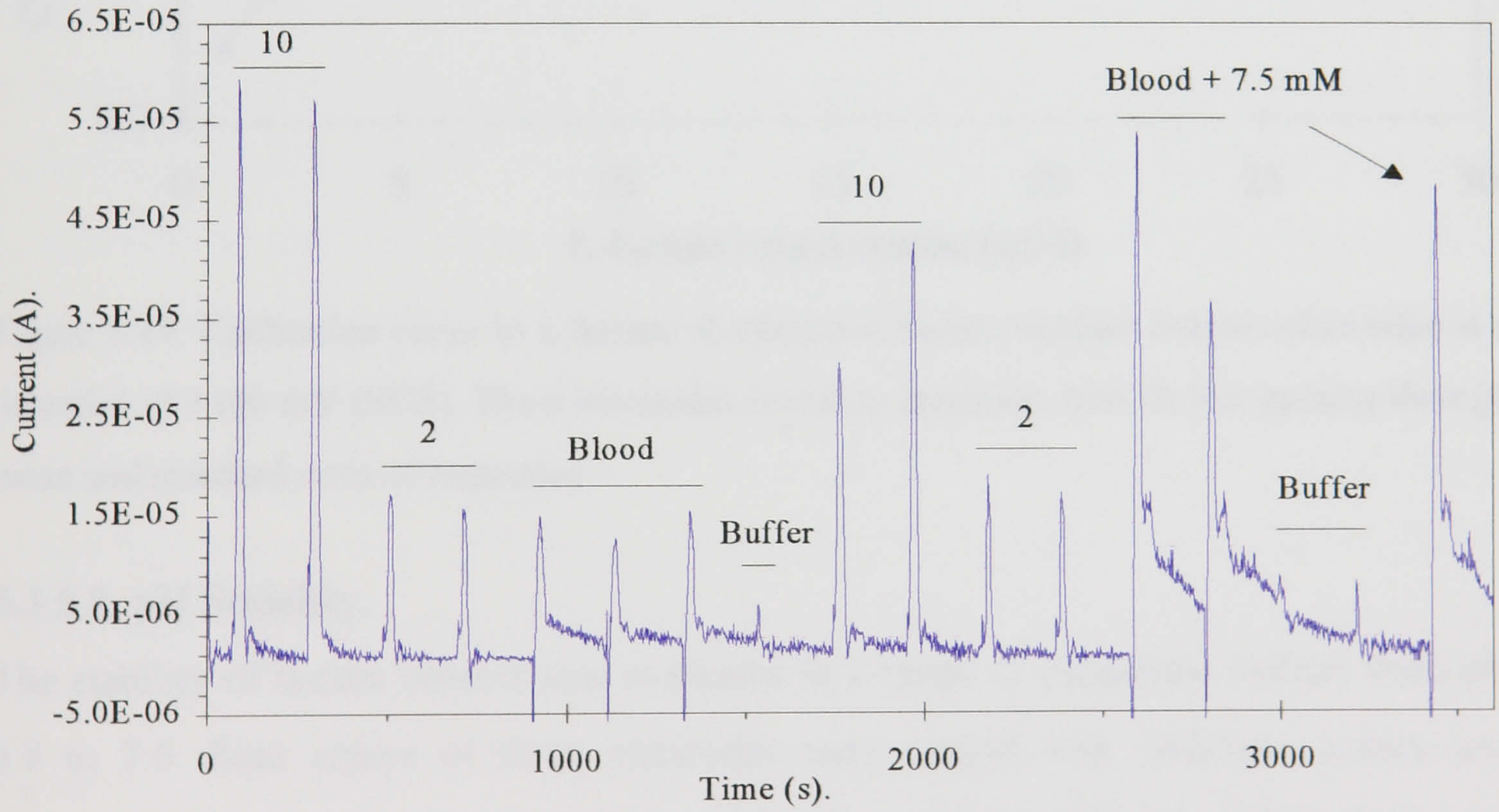


Figure 5.13. Trace of flow injection analysis of whole human blood samples using a modified three electrode sensor with a cellulose acetate coating (2% w/v in acetone). Working electrode modified with rhodinised carbon and lactate oxidase. Numbers represent concentration of L-lactate in sample (in mM), buffer and blood injections are as marked. The Ciba Corning blood analyser gave a reading of 1.1 mM lactate concentration in the whole human blood.

5.3.5. Assessment of Sensors with a Combined Membrane Structure.

5.3.5.1. Calibration of Screen-Printed Miniature Electrodes.

Miniature electrodes, coated with a dual membrane system, were assessed for L-lactate detection in batch mode. Two layers of cellulose acetate (2% w/v in 1:1 cyclohexanone to acetone) and one outer layer of polyurethane (1% w/v in 98:2 tetrahydrofuran to dimethyl formamide) were applied to the working surface. Figure 5.14. shows the calibration to L-lactate at +400 mV (SCE) of three electrodes tested two times. The sensors are linear from 0 – 3 mM with a sensitivity of 44.8 nA.mM^{-1}. The coefficient of variation at 2 mM L-lactate was only 19.8%, whereas the baseline current of the electrodes varied more (CV 108%).

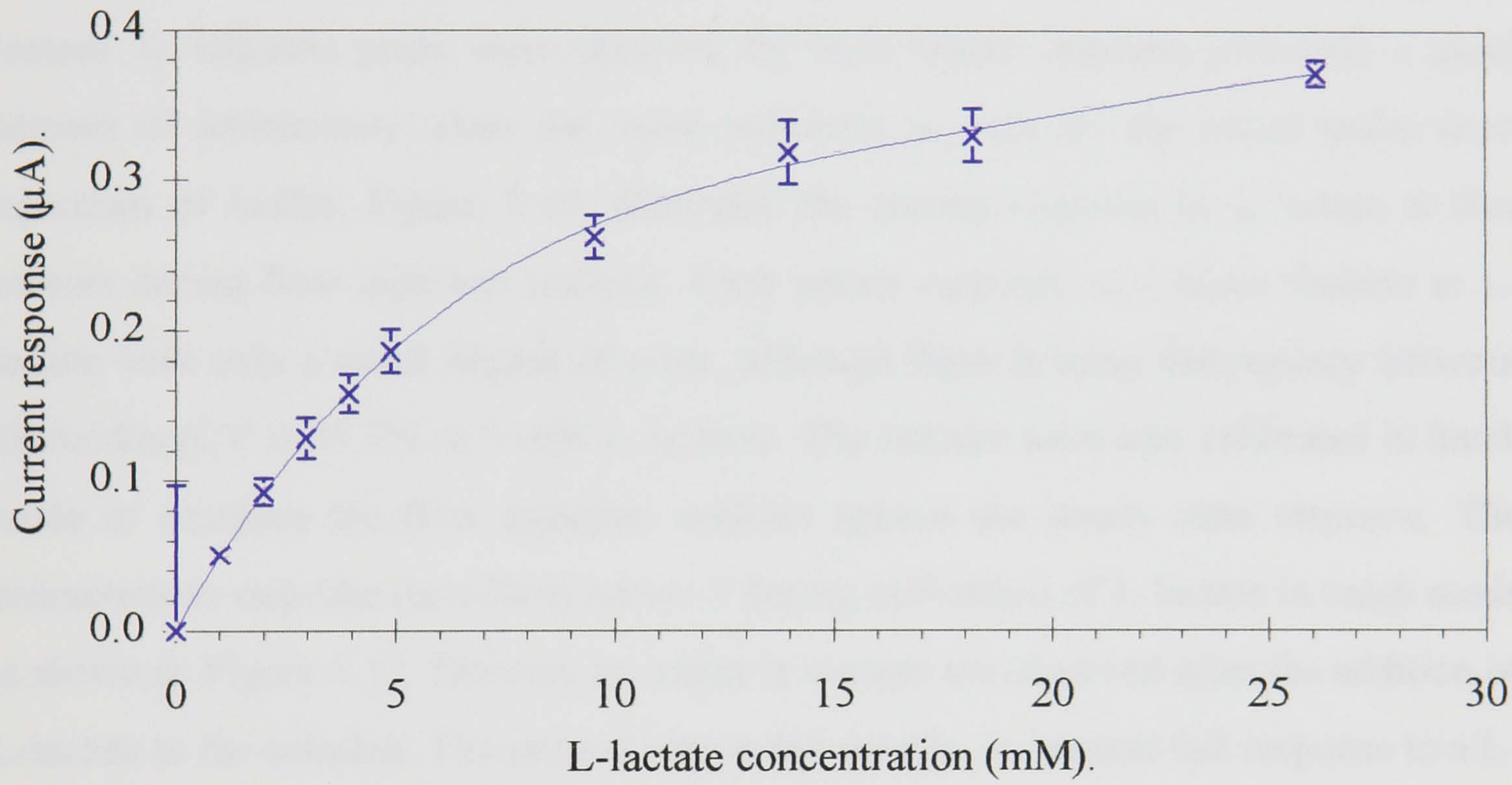


Figure 5.14. Calibration curve to L-lactate at miniature lactate oxidase enzyme-electrodes at a potential of +400 mV (SCE). Three electrodes tested in duplicate, best fit line passing through mean and standard error of responses.

5.3.5.2. pH Stability.

The stability of lactate sensors was evaluated in a range of phosphate buffers from pH 6.8 to 7.6. Four arrays of three electrodes were coated with cellulose acetate and polyurethane using the Cavro device and batch tested in triplicate. They gave very stable responses in all solutions (the coefficient of variation between all electrode responses at 10 mM L-lactate was 24.0%) with a linear range of 0 – 10 mM L-lactate and average sensitivity of 0.455 µA.mM^{-1}. The coefficient of variation between the slopes of the electrodes in different pH buffer solutions was 2.6%.

5.3.5.3. Screen Printed Sensors for Flow Injection Analysis.

Three electrode array sensors for use in a flow injection system were coated with a combined membrane structure of cellulose acetate (two layers applied to the working electrode, 2% w/v in 1:1 cyclohexanone to acetone) and polyurethane (one layer applied across all three electrodes, 4% w/v in 98:2 tetrahydrofuran to dimethyl formamide) using the Cavro device. These sensors were assessed for lactate detection in plain buffer, plasma and blood during flow injection analysis. They were also calibrated with L-lactate in buffer in batch mode.

Figure 5.15. shows the trace obtained during the calibration of a sensor using FIA (sensor 3). Discrete peaks were observed for each lactate injection with only a small amount of interference when the valve switched, as seen by the initial peaks from injections of buffer. Figure 5.16. illustrates the current response to L-lactate at five sensors during flow injection analysis. Each sensor responds in a linear fashion to L-lactate with only a small degree of error, although there is some discrepancy between electrodes (CV is 47.7% at 5 mM L-lactate). The sensors were also calibrated in batch mode to compare the flow injection analysis against the steady state response. The characteristic step-like trace from sensor 3 during calibration of L-lactate in batch mode is shown in Figure 5.17. Discrete increases in current are observed after the addition of L-lactate to the solution. The sensors responded quickly, in general full response to a L-lactate addition was gained after 60 s. The calibration to L-lactate of all five sensors in batch mode were plotted in Figure 5.18. Sensor 1 was calibrated three times and showed very reproducible responses. This, along with previous sensor stability results, meant that sensors 2 to 5 were only calibrated once in batch mode. The sensitivity, linear range, detection limit and coefficient of variation (at 5 mM L-lactate) of each sensor during flow injection analysis and batch tests are listed in Table 5.3. The sensitivity of the sensors was doubled when tested in batch mode which is reflected by the linear range of the sensors being longer during FIA. There was a slight variation in detection limit, most of the sensors had a lower detection limit when tested in batch but the detection limit of sensor 5 was lower in the flow injection system than when tested in batch mode.

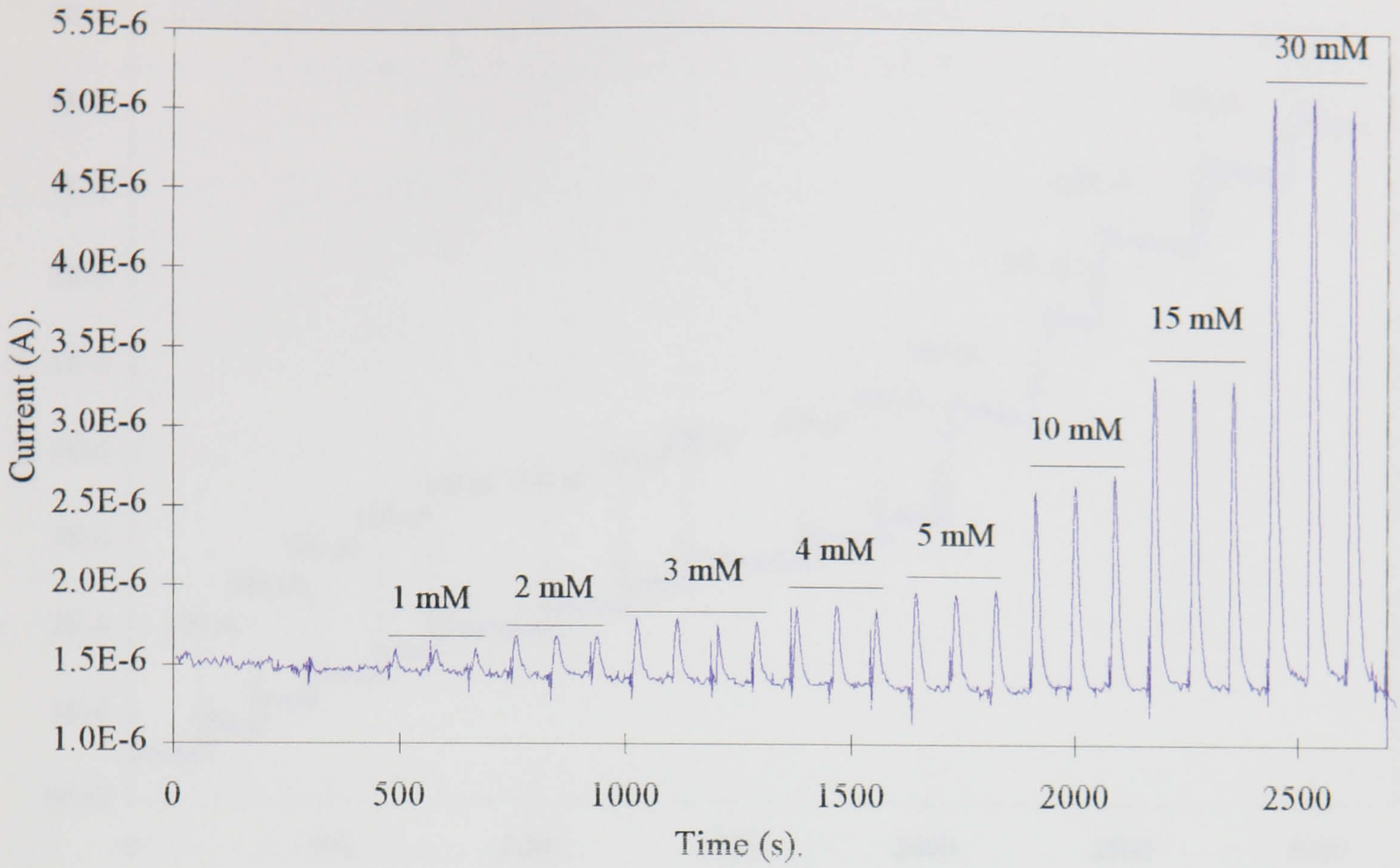


Figure 5.15. Calibration with L-lactate of a three electrode array sensor using flow injection analysis at +400 mV (Ag/AgCl). Flow rate 0.75 ml.min^{-1} with 1 ml between each injection, L-lactate concentration of samples indicated above injections.

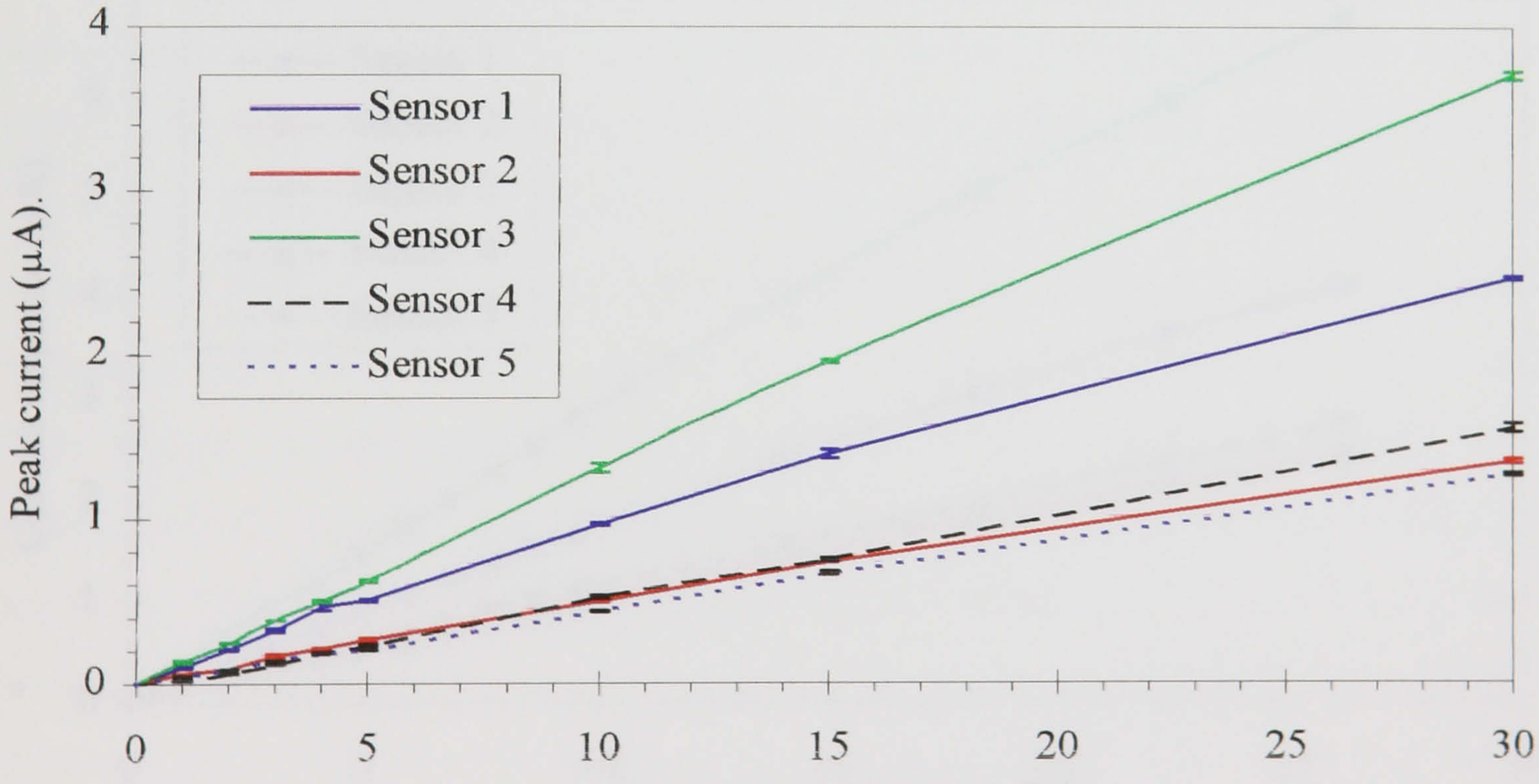


Figure 5.16. Calibration plots of flow injection analysis using three electrode array sensors. Conditions as in Figure 5.15, three injections at each concentration, five sensors individually tested.

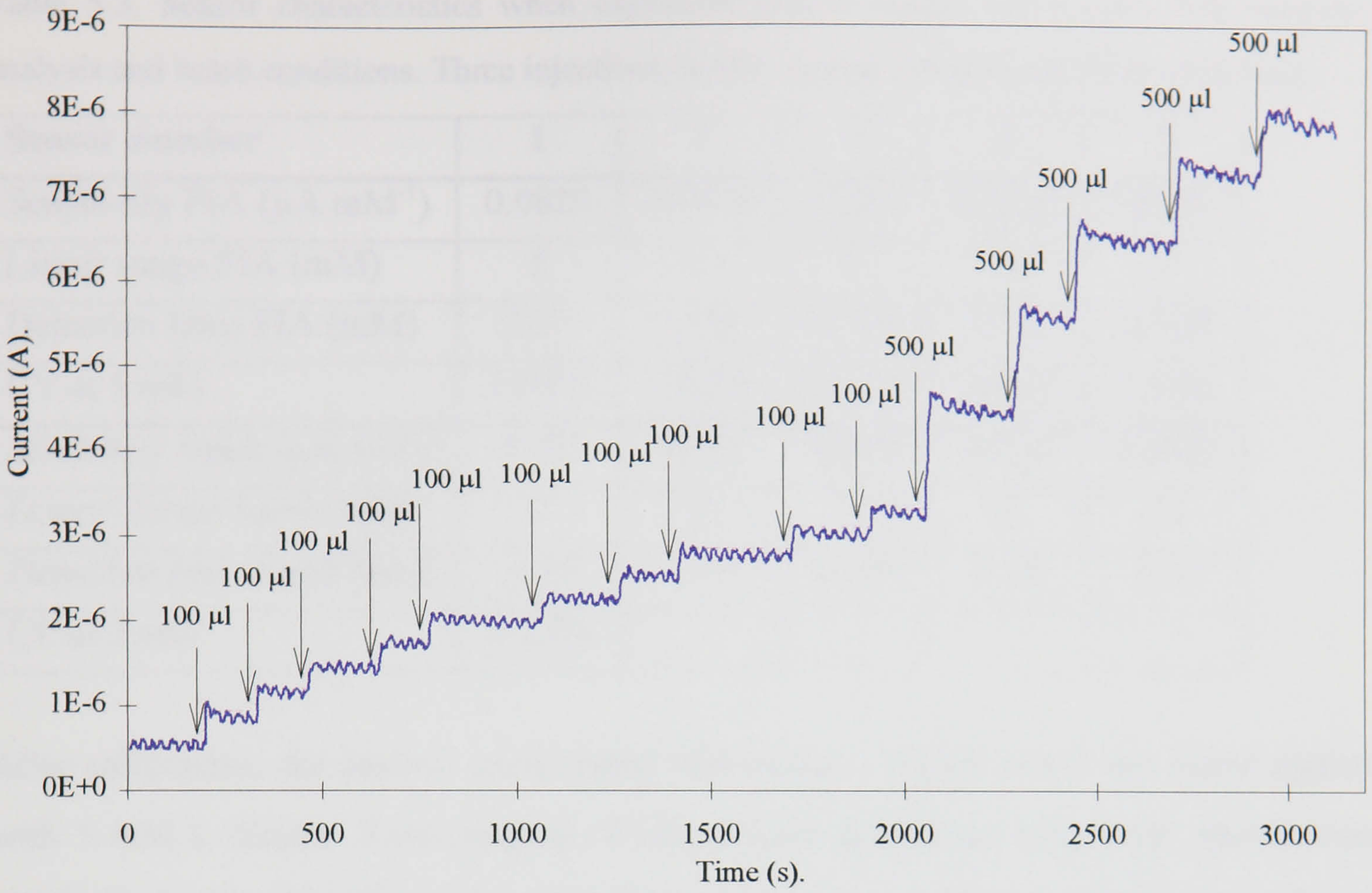


Figure 5.17. Response of three electrode array sensors to L-lactate as a batch measurement at +400 mV (Ag/AgCl). Volumes indicate amount of 0.2 M L-lactate solution added to 20 ml stirred buffer.

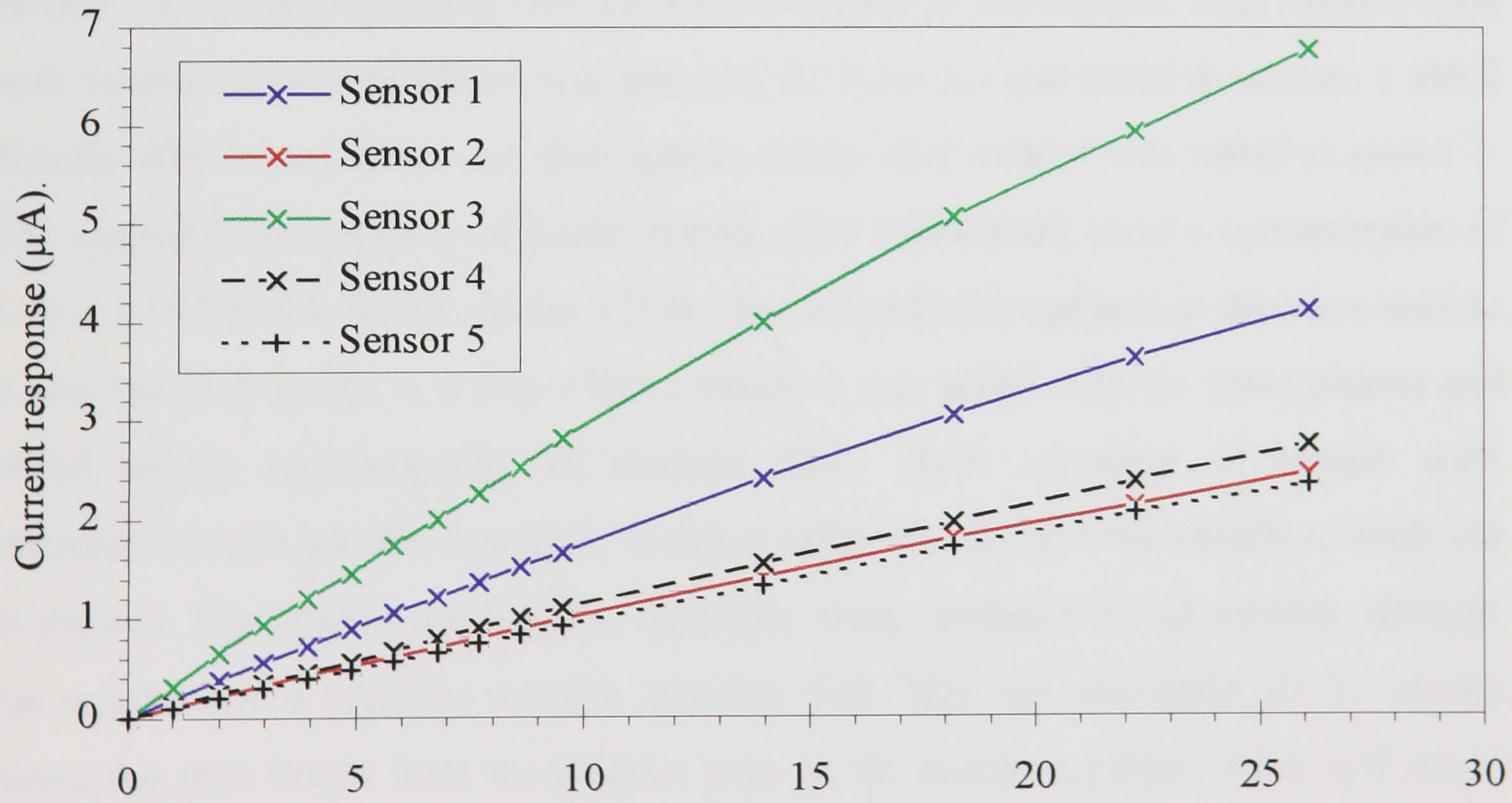


Figure 5.18. A plot of L-lactate calibration at three electrode array sensors tested in batch mode. Conditions as in Figure 5.17., five sensors individually tested.

Table 5.3. Sensor characteristics when calibrated with L-lactate under both flow injection analysis and batch conditions. Three injections in FIA, sensor 1 underwent three batch tests.

Sensor number	**1**	**2**	**3**	**4**	**5**
Sensitivity FIA ($\mu A.mM^{-1}$)	0.0820	0.0449	0.124	0.0524	0.0423
Linear range FIA (mM)	5	15	5	10	15
Detection limit FIA (mM)	1.71	1.20	0.755	1.22	0.709
CV at 5 mM	2.84%	7.12%	2.13%	5.81%	1.03%
Sensitivity batch ($\mu A.mM^{-1}$)	*0.182*	*0.103*	*0.306*	*0.116*	*0.0962*
Linear Range batch (mM)	*5*	*9*	*4*	*8*	*12*
Detection limit batch (mM)	*1.36*	*1.13*	*0.339*	*1.11*	*1.41*
CV at 5 mM	*9.15%*				

After calibration, the sensors were tested with plasma, whole blood and blood spiked with 5 mM L-lactate. Two samples of blood were used from which the plasma was obtained; the first blood sample was tested at sensor 1 to 3, the second at sensors 4 and 5. The actual plasma L-lactate concentration of whole blood was measured using a standard diagnostic spectrophotometric enzyme assay, the error associated with the readings arose from discrepancies in pipette volumes. There was a slight increase in the blood L-lactate concentration over the day, even though the samples were stored on ice with occasional mixing. Blood was analysed for L-lactate and tested at sensors 1 and 2 directly after it was taken, and then again 6 hours later when it was tested at sensor 3. The second blood sample, analysed directly after withdrawal, gave a concentration of 1.46 ± 0.075 mM L-lactate (mean $\pm$ S.D.) and the analysis repeated at the same time as it was tested at sensor 4, 8 hours later. Sensor 5 was tested with the same plasma and blood sample approximately 45 minutes later. Three injections of sample were interspersed with a buffer injection, to allow sufficient time for the sample to wash out of the cell. The baseline was regained with little, if any, hysteresis for all samples, although the sensors had a tendency towards baseline drift. This was accounted for by always measuring peak height from the baseline prior to the sample injection. After each set of samples, a regime of three injections of 5 mM L-lactate in buffer was carried out to check the sensor calibration for any decrease (or increase) in signal. The sensors were very successful in measuring L-lactate concentration, Table 5.4. outlines the data obtained.

Table 5.4. Data for individual sensors tested in FIA with plasma, blood and spiked blood. The response to 5 mM L-lactate in buffer was recorded before and after each sample, the subsequent response as a percent of the first response is shown. Conditions as in Figure 5.15., three injections of each sample.

Sensor number	**1**	**2**	**3**	**4**	**5**
Response to plasma (μA)		0.0850	0.287	0.0788	0.0967
Standard deviation (μA)		0.0057	0.034	0.0118	0.0100
Coefficient of Variation (%)		6.66%	12.0%	14.9%	10.4%
Calculated L-lactate (mM)		1.89	2.31	1.50	2.28
Actual plasma L-lactate (mM)		1.04	1.40	1.36	1.36
Standard deviation (mM)		0.125	0.095	0.120	0.120
Subsequent L-lactate response		63.6%	95.7%	97.3%	104%
Response to blood (μA)	*0.178*	*0.0700*	*0.301*	*0.0822*	*0.118*
Standard deviation (μA)	*0.00289*	*0.00520*	*0.0439*	*0.0117*	*0.00493*
Coefficient of variation (%)	*1.62%*	*7.42%*	*14.57%*	*14.26%*	*4.17%*
Calculated L-lactate (mM)	*2.18*	*1.56*	*2.42*	*1.57*	*2.80*
Subsequent L-lactate response	*100%*	*92.9%*	*100%*	*99.8%*	*57.8%*
Response to spiked blood (μA)	0.533	0.297	0.920	0.337	0.147
Standard deviation (μA)	0.0250	0.0180	0.0146	0.0164	0.0157
Coefficient of Variation (%)	4.70%	6.07%	1.58%	4.87%	10.7%
Calculated L-lactate (mM)	6.50	6.62	7.39	6.44	3.48
Actual spiked blood L-lactate (mM)	6.04	6.04	6.40	6.36	6.36
Standard deviation (mM)	0.16	0.16	0.14	0.16	0.16
Subsequent L-lactate response	101%	90.7%	92.8%	83.2%	62.1%

The sensors responded well to plasma, they gave reproducible current signals and the calibration to 5 mM L-lactate, post sample, showed that 3 out of 4 sensors retained their activity. Similarly, the current response to blood at the sensors was relatively reproducible and only 1 sensor lost a significant amount of response to 5 mM L-lactate. The sensors responded to L-lactate within blood, as shown by the increase in current to spiked blood over plain blood. Again, the error of the signal of each sensor was low and only sensor 5 lost an appreciable amount of sensitivity to 5 mM L-lactate in buffer. Sensor 2 actually regained most of its activity to 5 mM L-lactate after the blood analysis. These results are depicted in Figure 5.19. with the sensor L-lactate

measurement correlated to the enzyme test-kit L-lactate concentration measurement. The sensor signal reading was correlated to a L-lactate concentration by the calibration in FIA, and does not take any drop in sensitivity into account. From the intercept of the straight line equation of the trendline, it can be deduced that even if no L-lactate was present in the blood sample, the sensors would give a reading of approximately 0.86 mM L-lactate. The correlation coefficient of 0.9701 shows that there is a good correlation between the mean L-lactate values obtained from the two methods. The standard deviation of sensor L-lactate measurement also appears to be lower than that involved when using the diagnostic enzyme test-kit.

Control tests were also performed using sensor arrays without lactate oxidase. Four electrodes were used to observe the signal obtained in the presence of L-lactate, plasma and blood samples. Hydrogen peroxide was also tested on two electrodes before and after the plasma and blood samples to check if the response had dropped due to surface fouling. The data obtained is shown in Table 5.5. There was no signal to the L-lactate concentrations tested (up to 30 mM). The sensors clearly showed a signal in the presence of blood and plasma, on average the coefficient of variation for the blood sample was 10%. A drop in response to hydrogen peroxide of 18.2% was observed over the experiment for one of the sensors, whereas the other sensor signal dropped by 80%.

Table 5.5. Response of rhodinised carbon screen-printed array electrodes to hydrogen peroxide, blood and plasma at +400 mV (Ag/AgCl). Four electrodes coated with two layers of cellulose acetate (2% w/v in 1:1 acetone to cyclohexanone) and one layer of polyurethane (4% w/v in 98:2 tetrahydrofuran to dimethyl formamide) membranes.

Sample	**Mean ± S.E.**
Hydrogen peroxide (1 mM)	2.95 ± 0.58
Plasma	0.325 ± 0.080
Hydrogen peroxide (1 mM)	2.65 ± 0.41
Whole Blood	0.255 ± 0.034
Hydrogen peroxide (1 mM)	1.25 ± 0.25
Blood spiked with 5 mM L-lactate	0.325 ± 0.038
Hydrogen peroxide (1 mM)	1.10 ± 0.11

When interference was taken into consideration for each sensor, by subtracting the expected signal from the actual signal, the correlation between the sensor and the calculated L-lactate concentrations improved. The values obtained for blood measurement are shown in the correlation plot illustrated in Figure 5.20. Only the spiked blood analysis of sensor 1 was included since the blood measurement was used to calculate the interference, using the test kit result as the L-lactate concentration in whole blood. The spiked blood analysis at sensor 5 has been eliminated due to the uncharacteristic response, the 5 mM L-lactate standard before and after showed there to be a considerable drop in response. The plot displays a direct relationship between the sensor value and the calculated value (based on the test kit result) obtained for the L-lactate concentration within blood. The coefficient of 0.9823 means that there is a very good correlation between the two measurements.

A brief statistical analysis was carried out on the data obtained. An F test showed that there was no significant difference between the variance of the sensor and the diagnostic test kit determinations of L-lactate in plasma. From this it was concluded that the lactate sensors were just as precise as the diagnostic assay in determining L-lactate in plasma samples. Student *t* tests were carried out on the analysis of plasma and blood L-lactate to illustrate the accuracy of the sensor measurements. There was a significant difference ($P<5\%$) between the sensor and diagnostic test-kit L-lactate determinations. However, once the interference was taken into consideration there was no significant difference between the sensor and the diagnostic test kit. This meant that the two sets of data may be considered homogeneous.

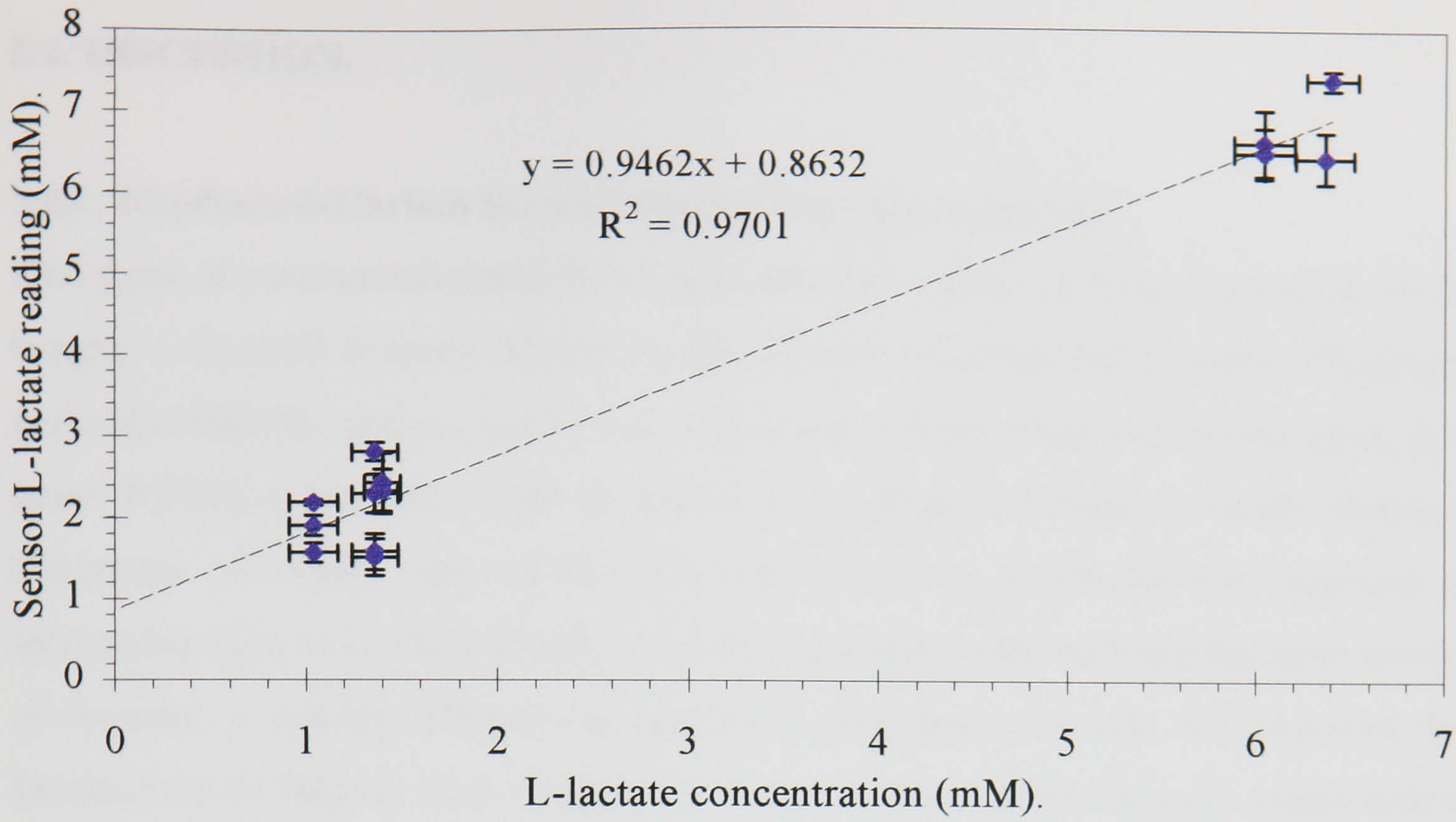


Figure 5.19. Correlation plot of lactate concentration values in plasma and blood obtained from screen printed lactate sensors compared to the calculated value from an enzyme spectrophotometric diagnostic kit estimate. Data from Table 5.4.

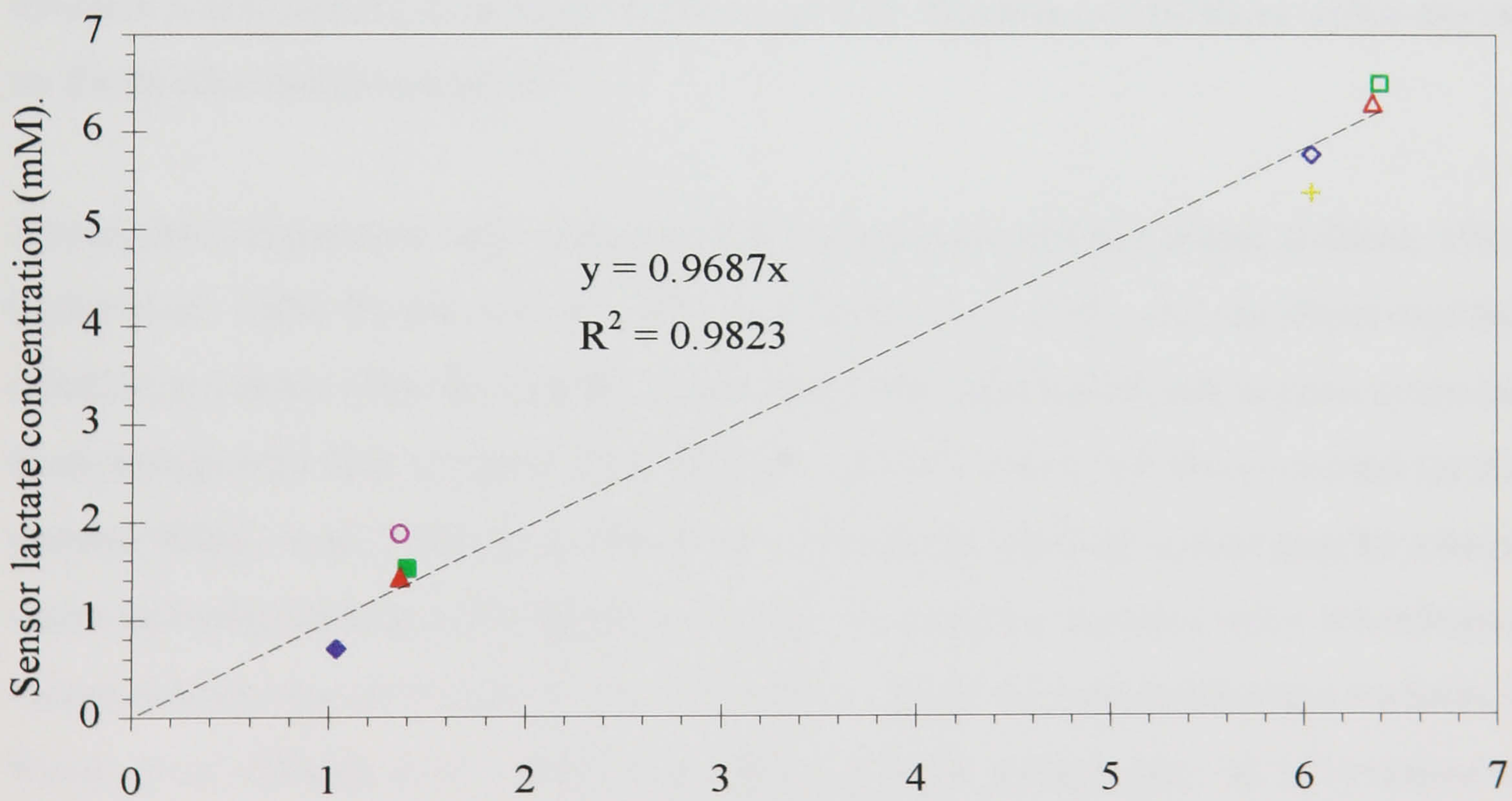


Figure 5.20. Correlation plot of L-lactate concentrations measured in blood once interference had been taken into consideration. Eight sensor measurements (from five sensors) compared to the corresponding calculated value which was based on a spectrophotometric diagnostic kit estimate.

5.4. DISCUSSION.

5.4.1. Rhodinised-Carbon Screen-Printing Ink Improvement.

Two types of commercially available rhodinised carbon with a rhodium content of 5% (by weight) were used to assess the best composition of metallised ink in terms of hydrogen peroxide detection and printing ability. An ink was sought which was easy to print, gave good definition and had a high sensitivity to hydrogen peroxide. Avocado Research Chemicals (Avocado) and Aldrich Chemical Company (Aldrich) both supplied an inexpensive form of rhodinised carbon and although they both contained the same amount of rhodium, a striking difference in response to hydrogen peroxide was observed. Ink formed from rhodinised carbon supplied by Avocado produced working electrodes with an even thickness and good pattern definition. The powder supplied by Avocado also printed more easily since it was less granular and it may have been due to the reduced particle size that the responses were much improved. A larger particle may not only be restricted through the screen during printing but will have a smaller surface area to weight ratio on which the rhodium could deposit. This would lead to a greater dispersion of rhodium within the ink on the smaller carbon particles.

Various ink compositions were made, based on ink formulas used by Cardosi & Birch, 1993; Collier *et al.*, 1996; Newman *et al.*, 1995, and White *et al.*, 1996, who used hydroxyethyl cellulose as a binder. This ink required a water insoluble outer-membrane to prevent the ink from pealing away from the electrode surface and cellulose acetate has been reported for this purpose (White *et al.*, 1996). It was therefore reasoned that cellulose acetate may be a better choice as binder since an outer membrane would no longer be required and a 4% cellulose acetate solution was used in this chapter to make an ink of the correct printing consistency. Wang *et al.* (Wang *et al.*, 1995) used 15% cellulose acetate (w/v in 1:2 acetone to cyclohexanone) to bind ruthenium on carbon and print on ceramic plates, which were subsequently dried at 15°C for 30 minutes, but this concentration would not have been suitable for our work. It was found in our study that the metallised carbon gave a more stable response and the inks printed with greater ease using hydroxyethyl cellulose rather than cellulose acetate as binder. These inks have the advantage of drying at room temperature (18-25°C) and require no curing at higher temperatures unlike other,

commercially available, screen printing inks (Wang *et al.*, 1996).

5.4.2. Improvement of Enzyme Screen-Printing Ink.

In order to ease the manufacturing process and reduce the time spent fabricating sensors, enzyme was incorporated into the metallised carbon ink to form a homogeneous catalytic layer. In previous chapters, enzyme inks were deposited using the method described Hart *et al.* (1996), whereby an enzyme ink was printed on top of the metallised carbon layer, enabling membranes to be applied between the layers to restrict the passage of interfering compounds. Lactate oxidase has a very low K_M (0.7 mM) which restricts the amount of L-lactate which can be present before the enzyme becomes saturated. A thick membrane is required to restrict the diffusion of L-lactate in order to detect it at clinically relevant concentrations. Therefore it was reasoned that a thick outer membrane of cellulose acetate could be applied to restrict the passage of both L-lactate and interferences. By using a homogeneous ink, therefore, not only would one catalytic ink need to be printed but the sensor fabrication would be eased by applying membranes in the final stage of fabrication rather than at two stages of production. The homogeneous ink was in fact more stable and gave a more linear response and a greater dynamic range to L-lactate than the two layers of inks. The increase in stability and linearity is possibly due to the immobilisation of lactate oxidase within the ink matrix since it has been reported that immobilised enzymes are generally more stable and they appear to be less susceptible to the normal activators and inhinitors that affect the activity of soluble enzymes (Guilbault *et al.*, 1991). The rhodinised carbon could be providing surface groups to which the enzyme adsorbed. This hypothesis was strengthened by the retention of enzyme activity on the second day of testing, in contrast to loss of activity after the first day with the ink made with lactate oxidase and carbon graphite, which may not have afforded such a good immobilisation matrix. Newman *et al.* (1995) also found that by combining the enzyme, glucose oxidase, with a metallised carbon ink, the stability and current response of their screen printed electrodes to glucose was improved.

By using the same amount of rhodinised carbon ink, the enzyme content was varied, allowing the comparison of different enzyme concentrations to be studied. A high concentration of enzyme would ensure high functional stability and allow the sensor to

produce higher signals (Pfeiffer *et al.*, 1992), but would be more expensive to produce. Conversely, a low concentration of enzyme would not be as expensive but may result in a low response to L-lactate. The best sensor performance was achieved using approximately 12.9 $U.cm^{-2}$ (or 10 mg lactate oxidase in 280 mg of rhodinised carbon ink). Pfeiffer *et al.* (1992) conducted a study of the relative sensitivity of lactate oxidase membranes containing different enzyme amounts and showed that the diffusion-controlled reaction was reached when using more enzyme than 1.6 $U.cm^{-2}$ membrane. They used an excess of this amount to ensure high functional stability, enzyme loading of 16 $U.cm^{-2}$, close to the value used in this chapter. It was found that by using a high enzyme concentration (20 mg lactate oxidase in 280 mg ink) the sensor response actually reduced. This may be due to the large enzyme molecule forming an insulating layer over the carbon particles of the ink and reducing the passage of both L-lactate and hydrogen peroxide, thus attenuating the signal. It has previously been reported that overloading the surface support with the biological component can decrease the activity due to restricted access to the active site (Barker, 1987) and a similar process is thought to be occurring here.

Lactate oxidase could be added to the ink in three ways: addition to the preformed ink, immobilised on the carbons first or dissolved into the hydroxyethyl cellulose first. Each method was tried; overall the addition of the enzyme to the preformed ink gave the most satisfying results in terms of reproducibility and linearity. The immobilisation of lactate oxidase on the carbons before forming an ink produced larger signals with a smaller linear range pertaining to enzyme in a free state. Similarly, when lactate oxidase was dissolved in hydroxyethyl cellulose first, the linear and dynamic ranges were reduced indicating that the enzyme was not as strongly immobilised, although the initial activity was higher than the electrodes containing enzyme mixed with ink. These effects are due to the way in which the ink constituents affected the enzyme. However, not enough information is available from the data presented here to provide a full explanation of the mechanisms occurring within the immobilisation matrix. Further investigations will have to be carried out to clarify the situation.

The polymeric binder, hydroxyethyl cellulose, was taken up for use in this thesis at 2% concentration (w/v) and has been used by many workers (Cardosi & Birch, 1993; Collier *et*

al., 1996; Newman *et al.*, 1995; White *et al.*, 1996). Hydroxyethyl cellulose is a nonionic, water-soluble polymer derived from cellulose and is used in the paint industry to thicken latex paint formulations to the desired viscosity (Burmeister, 1991). Hydroxyethyl cellulose thickeners influence the ink rheology primarily by thickening the aqueous phase, the formation of hydrogen bonds creates a concentrated network of cellulose chains. It has the ability to retain the ink components due to this structure and by using a higher concentration, it was reasoned that the enzyme and rhodinised carbon would be bound more tightly. This would possibly increase the current response since less hydrogen peroxide would be lost to the surrounding solution. In addition, the enzyme would be retained for a longer period of time within the matrix, taking longer to lose structural integrity (thus activity) or leech from the electrode. However, when 4% hydroxyethyl cellulose (w/v) was used as the binder, the ink did not print as successfully and there did not appear to be any improvement in the sensor response. The slight decrease in linearity and reproducibility when using 4% instead of 2% hydroxyethyl cellulose probably arose from the enzyme being more strongly bound (immobilised) within the matrix.

5.4.3. Membrane Application.

Since the commercialisation of an *in vivo* device would require it to be fabricated entirely by reproducible mass production techniques (Alvarez-Icaza & Bilitewski, 1993; Turner, 1993), an automated method for membrane deposition was sought. The ink-jet printer used earlier in the work required conducting salts to be added to the solution and a skilled operator, there also tended to be some error in membrane deposition due to the speed of the print head passing across the substrate (see Section 4.4.). A solution deposition device (Cavro) was employed to lay down the membrane by an automated mechanism similar to pipetting by hand. This novel technique for membrane application improved the reproducibility of sensor manufacture. However, the sensors with membranes applied by the Cavro printer were only slightly more reproducible than those with hand-pipetted membranes, probably because the error in the screen printing fabrication step limited the amount by which reproducibility of membrane application could be improved. The main advantage of this deposition technique was the speed and repeatability of membrane formation. Once the deposition pattern had been calculated and programmed into the microprocessor unit, an unlimited number of membranes could be applied, each containing

the same volume of solution in precisely the same position. Therefore the operator did not influence the way in which the membrane was laid.

5.4.4. Changing the Design of the Planar Sensor for Blood-lactate Monitoring.

The design of the sensors was altered to test the principle of manufacture and operation of sensors for *in vivo*, *ex vivo* and *in vitro* L-lactate monitoring. Inexpensive materials were used for the sensor construction so that the devices would be disposable and could be used for short periods of continuous operation. The required use life would depend on the patient monitoring time (typically 6 hours for surgical and 24 hours for critical care L-lactate monitoring (Siggaard-Andersen *et al.*, 1994)) and not be heavily influenced by the cost of the device. Very small enzyme electrodes were constructed for insertion in a catheter for use within a blood vessel. Kost and Hague (1996) suggest that an *in vivo* sensor must typically fit through a 20-gauge radial artery catheter, and allow withdrawal of blood and infusion of other solutions during monitoring. The planar electrode allowed not only a thin and flexible device to be constructed, thus helping to prevent turbulent flow, but also allowed the counter/reference electrode to be printed on the reverse side. This meant that the whole sensor could be integrated into a thin, flexible and yet robust device which would serve for *in vivo* measurements. Size, shape and rigidity of an in vivo sensor may play an important role in the biocascade response; a small and flexible sensor has been reported to be preferable (Reach & Wilson, 1992).

For *ex vivo* or *in vitro* L-lactate measurement, a flow injection system was to be used. This would allow automated continuous measurements (giving repeatability with easy operation and handling) which would require a small sample volume which could be interspersed with washing steps to reduce sensor fouling. It also meant that the blood was contained, thus reducing the risk of infection to both the operator and patient. The sensors were built to a practical size using a three electrode array which would prevent the deterioration of the reference electrode thus creating a more stable response.

5.4.5. Assessment of Membrane Operation in Plasma and Blood.

The composition of the cellulose acetate membrane had already been improved in Chapter 4, to limit the amount of interferents and L-lactate which penetrate to the sensing surface

below. From the earlier studies on the affect of a protein solution on sensor responses of L-lactate, polyurethane was chosen as the outer membrane (Section 4.4.). However, the membrane structure had not been improved for operation in plasma and blood samples, nor have mass fabrication techniques been applied. The initial plasma and blood tests using the sensors in the flow injection system were undertaken to assess the ability of polyurethane membranes to prevent fouling of the sensor surface and to inhibit the passage of interferents to the electrode surface. Batch tests were not carried out due to the hazard involved in handling and disposing of such large quantities of blood needed for the experiments. The sensors for use in the flow injection system were dip coated with membranes because the Cavro device had not been programmed to deposit over the three electrodes and this was the quickest method which would cater for the need of the initial experiments. The original concentration of polyurethane (4% w/v) was used and a lower concentration (2% w/v) was tried in order to check if the sensitivity would be greater, but still protect the sensor from fouling. Cellulose acetate was tested as a comparison for the fouling experiments and to study its effect on limiting the passage of interferents.

All membranes worked adequately in plasma and allowed the sensor to detect L-lactate without becoming too fouled, although the cellulose acetate provided more protection against interferents, and gave a more accurate L-lactate concentration. The 4% polyurethane inhibited fouling of the sensor in blood more than the other membranes, but did not provide protection against other oxidisable compounds. Unfortunately, the response to L-lactate was not significantly increased when using 2% polyurethane membrane and the sensors showed less protection against fouling. Polyurethane has generally been used at concentrations above 4%, but mainly because this extends the sensor linearity (Churchouse *et al.*, 1986; Vadgama *et al.*, 1989). Sternberg *et al.* (1988) used two dip coats of 2% polyurethane to coat needle glucose sensors and extend their linear detection range. When tested *in vitro* and *in vivo*, glucose measurements were possible for 2-6 hours, although they did not compare with higher polyurethane concentrations. Sternberg *et al.* concluded, however, that the reproducibility of outer layer deposition was definitely the weak link in the microsensor preparation. This highlights the need for an automated method of membrane application to enhance the reproducibility of the sensing device.

The cellulose acetate membrane gave an accurate and stable response in blood, although, subsequently, it did not respond as well to spiked blood. The sensor recovered its activity towards L-lactate after subjection to blood but when spiked blood was introduced, the signal dropped significantly and was not fully regained there after. It can be assumed that the membrane was harmed in some way and deteriorated further when blood was reintroduced. Further tests would have to be carried out to determine the cause of the drop in response, but it is known that when cellulose acetate is used as dialysis tubing, blood reactions take place, leading to complex protein adsorption (Cornelius & Brash, 1993).

5.4.6. Testing the Strategy for Improving Sensor Performance by Using A Combined Membrane Structure.

Since it was shown that polyurethane was a good membrane choice for its antifouling properties and cellulose acetate was better at excluding species which interfered with the L-lactate signal, a combination of both membranes was reasoned to afford the sensor with both properties. The most commonly used membrane for implantable biosensors is polyurethane (Pickup & Thevenot, 1993) and although many other workers have previously used cellulose acetate and polyurethane membrane layers (Bindra *et al.*, 1991; Brunstein *et al.*, 1989; Hu *et al.*, 1993; Mascone *et al.*, 1993; Pfeiffer *et al.*, 1993; Sternberg *et al.*, 1988; Zhang & Wilson, 1993), this is the first study of the affect of the individual membranes on L-lactate sensors in buffer, plasma and blood.

Initial tests of the miniature electrodes had previously shown, as expected, that the current signal, and therefore the sensitivity, was small. A membrane structure comprising of a layer of 2% cellulose acetate and an outer layer of only 1% polyurethane was used so that the combined features of the membranes would limit the interference and reduce the passivation by blood, but not reduce the signal to undetectable amounts. This reasoning worked, the sensor was linear up to 3 mM with a sensitivity of approximately 45 $nA.mM^{-1}$, but it was not known how the polyurethane outer membrane would affect the response in biological fluids.

Under normal conditions, arterial blood has a pH of 7.39 to 7.44 (Long *et al.*, 1971) but blood hydrogen ion concentration compatible with life can vary from pH 6.8 to 7.8 (Pruden *et al.*, 1987). This would necessitate the sensor be capable of operating over a narrow pH

range. Sensors coated in both cellulose acetate and polyurethane were shown to operate in a stable manner to L-lactate in buffer solutions of pH 6.8 to 7.6, which covers the main range of blood pH. Immobilised lactate oxidase has also been shown by other workers be stable in the range between pH 5 and 8 (Mullen *et al.*, 1986; Pfeiffer *et al.*, 1992; Wang & Liu, 1993).

Flow injection analysis was used to assess the ability of the three electrode sensors coated with cellulose acetate (2 layers of 2% w/v) and polyurethane (1 layer of 4% w/v) to detect L-lactate. A very repeatable signal was achieved to each L-lactate concentration along with good linearity. The dynamic range was very wide; the sensors detected at and above the clinically relevant range of L-lactate. The sensors could be easily calibrated within the flow system so there was no problem with the variation of current signal between sensors. There was a small drop in current response, but again, they could be easily calibration in line. The sensors were also calibrated in batch mode, to check whether or not they could be used as one shot devices. They gave a slightly shorter linear range but a higher sensitivity to L-lactate than during flow injection analysis and were very acceptable to be used in this detection mode.

5.4.7. Determination of Blood L-lactate.

It was noticed that the blood sample L-lactate concentrations increased through the day. This has been previously reported and rises due to anaerobic glycolysis (Caraway & Watts, 1987; Harris *et al.*, 1996). Samples should be stored on ice and analysed as soon as possible to prevent the L-lactate rising. However, the L-lactate level is stable in the plasma phase once it was separated from the cells.

Blood samples analysed at sensors with combined membrane structure or with a diagnostic enzyme test-kit, showed good correlation and a statistical F-test indicated that there was no significant difference between the precision of the two methods. The best fit linear line through the points showed an intercept relating to an additional measurement at the sensors. When electrode arrays were used, in the absence of lactate oxidase, there was a small response to blood, indicating that interferences were still passing through the membrane structure and reacting at the electrode surface. This additive interference was eliminated by

subtracting the anomalous current from the response. Once this manipulation had been performed, no statistical difference between the sensor and test-kit responses could be found.

There are several L-lactate detection methods available on the market, which are detailed in Table 5.5. along with the L-lactate sensor developed in this thesis. From the table it can be seen that if the device was to be used in a flow injection system, then it would compete with the Chiron 800 Series. There are presently no devices which operate *ex* or *in vivo*, therefore this device could satisfy a niche market if it was capable of passing clinical trials.

5.4.8. Conclusions.

For a sensitive and stable L-lactate screen-printed electrode, the best ink consisted of lactate oxidase incorporated into a preformed ink of rhodinised carbon, carbon graphite powder and hydroxyethyl cellulose binder (2% w/v in buffer). Neither cellulose acetate or polyurethane alone could prevent interferents and fouling but a combination of both membranes afforded the sensor with the advantages of each individual membrane. Mass fabrication techniques could be used for the whole sensor construction. The results show that mass fabricated enzyme electrodes could be used to precisely determine the L-lactate levels in blood and plasma using a flow injection system.

Table 5.6. A comparison of various L-lactate detection systems.

System	Method of Detection	Substrate concentration range	Response time (throughput)	Comments
Stat plus, Yellow Springs Instrument Company	Electrochemical Lactate oxidase immobilised at Pt anode, hydrogen peroxide detected amperometrically	up to 30 mM	45-65 s (40/hr)	Sample volume 25 μl Sample diluted
800 series, Chiron Diagnostics	Electrochemical Printed four electrode system with lactate oxidase. Amperometric detection of hydrogen peroxide	0-30 mM Resolution 0.01 mM	90 s to reading (25/hr)	Flow system to analyse a range of blood components
LM5, Analox	Electrochemical Amperometric detection of rate of oxygen uptake by lactate oxidase	0-10 mM 5 μl sample 0-20 mM 2.5 μl sample	20 s	Controlled temperature Sample diluted
Lactate Analyser 640, Roche Bio-electronics	Electrochemical Amperometric detection of hexacyanoferrate mediated cytochrome b_2 reaction at a Pt anode	0-12 mM	2-3 minutes	Dilution step required
Sigma Diagnostic Kit, Sigma Diagnostics	Spectrophotometric Lactate oxidase and horseradish peroxidase with chromogen precursors, absorbance read at 540 nm	0-13 mM	5-10 minutes	*In vitro* diagnostic use Have to centrifuge sample
Accusport, Boehringer Manheim (New Zealand)	Reflectance photometry Lactate oxidase and mediator reaction observed at 660 nm	0.8-22 mM blood 0.7-26 mM plasma	60 s	Capillary blood from fingertip or earlobe, sample volume 15-50 μl
Sensor array in flow cell, Cranfield Biotechnology Centre	Electrochemical Printed three electrode array, amperometric detection of hydrogen peroxide generated by lactate oxidase	0-30 mM	20 s	No dilution step Sample volume 200 μl

CHAPTER 6:

GENERAL CONCLUSIONS AND SUGGESTIONS FOR FUTURE WORK.

The main purpose of this work was to develop a mass-producible amperometric enzyme electrode employing lactate oxidase for *in vitro* use. Chapter 1 discussed the need for continuous monitoring of lactate, both in clinical and sports diagnosis, and the present methods of lactate determination were outlined. The principles of electrochemical techniques were described leading to a survey of the current stage of development of enzyme probes that utilise one of four main lactate oxidising enzymes.

The first experimental chapter (Chapter 2) assessed the value of a number of different surface chemistries as possible amperometric transducers for the detection of L-lactate using lactate oxidase, and investigated the suitability of mass fabrication techniques to each chemistry. Advantages of carbon, including its low cost, malleability to different fabrications and modifications and practical use as an electrode, lead to various lactate measurements being attempted at modified forms of carbon electrodes. Novel and traditional chemistries of hydrogen peroxide detection, mediation and direct communication between the enzyme and the electrode were chosen for their ability to lower the applied potential of the enzyme electrode and increase the selectivity of detection. Hexacyanoferrate film, Prussian Blue and rhodium were used to modify carbon to electro-catalytically detect hydrogen peroxide. Tetrathiafulvalene was used as a mediator between lactate oxidase and carbon electrodes, including screen-printed electrodes. Polypyrrole, either in micro-tubules or coated onto latex beads, was used to immobilise lactate oxidase, and the possibility of direct electron transfer to the underlying carbon electrode was explored. For the purpose of this project, only one approach was considered for the development of an *in vivo* amperometric enzyme electrode. Although the novel chemistries of hexacyanoferrate films and polypyrrole gave responses to lactate in the presence of lactate oxidase at low potentials, further work is needed to afford the sensor with stable and sensitive signals comparable to the rhodinised carbon. Rhodinised carbon was selected for further investigation and development because it was: amenable to mass-fabrication techniques, easy to handle, stable both during storage and testing and gave a sensitive response to hydrogen peroxide.

The signal of other noble metal-modified carbons to hydrogen peroxide were examined in Chapter 3, including palladinised and platinised carbons. The examination revealed that rhodinised carbon was most suited for hydrogen peroxide detection at low potentials since

it gave the greatest sensitivity. A series of rhodinised carbons were evaluated to ascertain the best formulation in terms of catalytic properties and printing ease. It was found that the relationship between the rhodium and carbon is complex, and greatly influences the catalytic behaviour of the combined materials. Electroplating rhodium gave a different response to hydrogen peroxide than screen printed rhodinised carbon powder (Chapter 3) and even two carbon powders with the same rhodium content (by weight) gave very different responses to hydrogen peroxide after being screen printed in similar inks (Chapter 5). This relationship requires further exploration to attain a greater understanding of the requirements for specific catalytic behaviour of rhodinised carbon towards hydrogen peroxide.

It was shown that the electrochemical interference from ascorbic acid was lower at screen-printed rhodinised-carbon electrodes poised at potentials above +300 mV (SCE) than unmodified-carbon electrodes, but was higher at lower potentials. Interference from oxygen was also present at screen-printed rhodinised-carbon electrodes, at -100 mV (SCE), therefore the operating potential was restricted to positive values. A working potential of +400 mV (SCE) was chosen to catalytically detect the enzymic generation of hydrogen peroxide in the presence of lactate and oxygen. Fortunately, lack of oxygen did not affect the lactate signal at +400 mV (SCE) and this was thought to be due to the entrapment of molecular oxygen within the sensor matrix.

It was acknowledged that membranes would have to be employed to increase the linearity of lactate detection and decrease interference from other oxidisable species. A systematic study in Chapter 4 of cellulose acetate concentration, solvent composition and membrane thickness showed that the optimum membrane conditions were achieved with 2% cellulose acetate (weight for volume) in a 1:1 mixture of acetone to cyclohexanone. This limited the passage of both lactate and ascorbate, but to different degrees. Little interference from ascorbic acid was observed and the linear detection range of lactate was increased to above the clinically relevant range. In Chapter 4, Nafion was demonstrated to effectively limit anionic species; not only did it exclude ascorbic acid but the enzyme electrode response to lactate was also considerably reduced, indicating that the passage of lactate was restricted to a similar extent. The thickness and formula of membranes requires further development to allow the significant levels of L-lactate to be accurately determined with negligable

electrochemical interference from other species. Alternative membrane materials could also be tested, such as polycarbonate.

Furthermore, membranes are likely to be required at the enzyme electrode surface for progress towards implantation by providing a biocompatible surface that is also resistive to the fouling by protein adsorption which lowers the sensor sensitivity. A comparative test of Pellethane, cellulose acetate and MPC-co-BMA in buffered albumin solutions showed the polyurethane to be most effective at preventing protein adsorption, although the sensitivity of the enzyme electrode was compromised. Although Pellethane is known to be a worthy membrane for implantable devices (Lelah & Cooper, 1986), newer polyurethanes which have more favourable properties for *in vivo* devices were not available for this study. However, it would be interesting to employ the new membranes to see if they improved the sensor response when continually analysing blood. MPC-co-BMA showed a revival of response in buffer after storage in buffered albumin solution, indicating that reversible protein adsorption occurred. This interesting membrane material requires further research in the capacity of an outer coating for a sensor requiring anti-fouling capabilities.

A common theme throughout this thesis has been the application of mass-fabrication techniques to construct the sensors. A number of printing technologies have been exploited to fabricate low cost, reproducible lactate oxidase-modified carbon electrodes. In Chapter 2, screen printing displayed a high degree of reproducibility; more so than hand fabricated electrodes, and was therefore used extensively in this study. The ink-jet printer is a useful tool for the deposition of small volumes of solubilised material. Unfortunately the ink-jet printer used in this study was not reproducible enough to enable sensors to be fabricated using this technique. Instead, a Cavro printer was employed, in Chapter 5, to deposit known volumes of solution down to nano-litre amounts in a programmable and repeatable manner. Other advantages of this technique included its simplicity, ease of operation and speed.

A quick and simple modification procedure, suitable for one step printing onto screen-printed electrodes was achieved by using a homogeneous ink. The empirical development of this ink, described in Chapter 5, combined lactate oxidase with an ink based on rhodinised carbon in hydroxyethyl cellulose solution. An ink consisting of carbon graphite powder,

rhodinised carbon from Avocado Research Chemicals Limited (Lancaster), hydroxyethyl cellulose (2% w/v in phosphate buffer) and lactate oxidase in a weight ratio of 2:8:18:1 was the most suitable.

To investigate the behaviour of the lactate sensor in physiological solutions, a flow injection system was employed. This approach also provided some insight to the requirements of the membrane structure for a possible *in* or *ex vivo* continuous monitoring device. A strategy was developed for limiting both the passage of interfering species and the adsorption of protein by using a combination of cellulose acetate and polyurethane membranes applied in layers. Again, flow injection analysis of spiked buffer, whole blood and plasma was carried out. The planar screen-printed arrays detected L-lactate sensitively and accurately at +350 mV (SCE) with little fouling of the electrode surface. Fouling could, in future, be further reduced by extra washing steps and the addition of cleaning agents to the buffer stream. The responses to lactate were precise once a small interfering signal had been subtracted. The interference was also noticed on sensor arrays lacking lactate oxidase. In future, to eliminate these interfering signals, another working electrode without enzyme could be included in the array, to electronically subtract its signal from the other working electrode. The addition of a proteinous compound like bovine serum albumin would have to be added, in place of the enzyme, so that the two electrodes would be similarly insulated by proteinous material.

To achieve the goal of implantable sensors, the sensor design was altered to enable the enzyme electrode to pass down a catheter. Flexible material was adopted as the base substrate onto which the enzyme electrode was screen printed. Cellulose acetate and polyurethane membranes were applied by a Cavro printing device to encapsulate the working surface. The sensors were operated at +400 mV (SCE) in a stirred buffer solution and calibrated with L-lactate. It was envisaged that with a silver/silver chloride combined reference and counter electrode on the reverse, these sensors could be used for L-lactate determination in whole blood. Further improvements to the membrane structure and reproducibility would need to be carried out before *in vivo* tests could be carried out.

It may be possible in future studies to add a stabilising compound which will enable the sensors to operate in solutions for a longer period of time and may enhance their stability

under dry storage conditions. Polymerized alcohols and carbohydrates have been shown to be suitable materials for the long-term stabilization of immobilised enzymes. Two such compounds dextran and lactitol, have been added to a lactate oxidase paste, apparently stabilising the enzyme (Rohm *et al.*, 1996).

The work carried out in this thesis has drawn together various sensor technologies and techniques to construct L-lactate amperometric enzyme electrodes. Selection and improvement of rhodinised-carbon electrodes was undertaken. It has been shown that by using conventional biosensor technology it is possible to improve the response and reproducibility of an amperometric enzyme electrode by applying novel mass fabrication techniques. An innovative approach to sensor fabrication has lead to a miniaturised planar enzyme electrode that passes through a catheter and can linearly detect L-lactate concentrations up to 3 mM, within the clinically acceptable range. A planar sensor array for use in a flow system has also been fabricated entirely by mass production techniques and can accurately determine, after correction, the L-lactate concentration in plasma and whole blood.

CHAPTER 7:

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